FINAL REPORT

SURFACE WATER CONTAMINATION BY INSECTICIDES: DATA FROM AQUATIC INSECT TESTS THAT PERTAIN TO WATER QUALITY CRITERIA

by

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Introduction and Literature Review

One problem posed by chemical contamination of surface water is establishing a rational basis for regulating the use of chemicals so that benefits are attained without deterioration of water as a habitat for aquatic organisms and for human consumption and enjoyment. Generally, water that is a safe habitat for a diverse aquatic community is also safe for human utilization. A rational basis for regulating each chemical is derived from research data that are generated with aquatic organisms under natural conditions or those that simulate natural exposure.

Pesticides are one of the largest groups of chemicals for which water quality standards or criteria have been established (National Technical Advisory Committee to the Secretary of the Interior, 1968; NAS/NAE, 1972, U.S. EPA, 1977 and the Ohio EPA, 1978). Pesticides enter surface waters in the United States from manufacturing operations (industrial effluents), municipal sewage, agricultural use, forest treatments, other miscellaneous uses and by translocation from other environmental compartments. Water pollution by pesticides is a major concern of federal and state regulatory agencies and agencies responsible for the management and protection of our natural resources. Pesticide-free water systems are not compatible with our industrial-agricultural system of production, but it is possible to establish safe upper limits of concentration of each pesticide that is a potential contaminant of surface water. Insecticides are the group of pesticides that has been given the most attention in regard to allowable stream burdens. The setting of tolerances or limits (i.e., water quality standards and criteria) is based on biological data acquired with aquatic organisms, primarily fish and to a lesser extent, aquatic insects.

Acute toxicity data (24 or 96 LC_{50}) for aquatic organisms are a useful starting point for establishing standards or criteria. But pesticides approaching acutely toxic concentrations in streams would undoubtedly alter the ecology of the stream and may not be safe for human utilization. Hence, factors other than acute toxicity become

important elements in establishing criteria or standards for certain groups of pesticides. For instance, organophosphate insecticides comprise nearly 50% of all insecticides used in the United States today and their mode of action as poisons is the inhibition of acetylcholinesterase, an enzyme common and essential to metazoans. The inhibition is long-lasting (regeneration times are often 10 to 15 days) to such an extent that organophosphates are classified as irreversible inhibitors of acetylcholinesterase. Consequently, stream organisms exposed intermittently to sublethal concentrations of organophosphates may exhibit cumulative acetylcholinesterase inhibition, an effect that would not be revealed by bioconcentration studies with organophosphates since the latter do not typically accumulate in body tissues to any extent. Parathion, the toxicant used in this study, is representative of the organophosphate group. It is used extensively in agriculture (about 25% of all insecticide use in the U.S.) and has been detected in Ohio and U.S. rivers in U.S. EPA monitoring studies.

Another important aspect of toxicity for which very little research has been conducted is in the area of seasonal temperature fluctuations. Active populations of poikilothermic stream vertebrates and invertebrates exist throughout the year in Ohio streams. Seasonal changes of temperature from $0^{\circ}C$ to $30^{\circ}C$ are not uncommon. These changes would obviously affect uptake, mode of action, metabolism and excretion of xenobiotics. While the poikilothermic stream organisms may adjust biochemically to these great changes in temperature by utilizing several different strategies, it is unclear as to how this may affect their susceptibility to xenobiotics. Municipal and industrial discharges of toxic substances and run-off from agricultural areas occurs year-round. It is the purpose of this report to present some basic knowledge on the effects of season and temperature on the susceptibility of stream organisms to a common organophosphate pesticide.

Biochemical Adaptation to Temperature Changes

Environmental temperature changes pose severe problems for ectotherms in temperate climates. Survival mechanisms that have evolved to cope with seasonal variation include extreme changes in life cycles or spending dormant periods in areas protected from freezing temperature (i.e., below ground). However, aquatic habitats are never completely frozen (particularly streams) and ectotherms existing in these areas (mainly fish, invertebrates and some amphibians) are known to remain active during winter months. Thus, organisms that must live at temperatures as high as 30°C in the summer and as low as 0°C in the winter would undoubtedly exhibit altered biochemical activity and/or the organisms may have mechanisms to compensate for seasonal temperature changes. Bullock (1955), in a review article, suggested that many poikilothermic organisms exhibit metabolic and physiological activity independent of temperature to varying degrees. He felt that this represents a compensation rather than an insensitivity of metabolic activities or the rate of various biochemical functions to temperature. In a more recent review article, Hochachka and Somero (1973) explained the various possible methods by which ectotherms possibly compensate for environmental temperature changes. They theorized that three major forms of compensation are utilized.

1) Evolutionary rate compensation - This method is accomplished over long geologic periods of time. Organisms using this method have usually adapted to one narrow range of temperature. The best example of this method is found in the arctic fish, Trematomus borchgrevinki, which lives at water temperatures of -2°C throughout the year, yet has metabolic activities and growth rates comparable to tropical fishes. Organisms adapted to such narrow temperature ranges usually cannot survive fluctuating temperatures and studies indicate that their enzymes have a very narrow temperature range of activity. Hazel and Prosser (1974), in a

more recent review article, surmised that such organisms have lost their capacity for thermal acclimation.

2) Seasonal rate compensation - In studies comparing an aquatic ectotherm species that was acclimated at different temperatures, organisms acclimated to the cold demonstrated much higher metabolic rates than would have been predicted by extrapolating warm-acclimated metabolic rates of the same species, assuming normal Q_{10} values. This was also true for metabolic rates of isolated tissues. Baldwin and Hochachka (1970), working with rainbow trout, discovered different isozymes of acetylcholinesterase in warm-and cold-acclimated trout, suggesting that seasonal isozymes exist in this species. Moon and Hochachka (1971, 1972) made a similar discovery in experiments with rainbow trout and isocitrate dehydrogenases. Hazel and Prosser (1974) cited evidence from other authors that biochemical acclimation to temperature may exist in Atlantic salmon, green sunfish, coho salmon, brown bullhead, earthworms, Rana pipiens (leopard frog) and P. brevicornis (an Alaskan beetle). Hochachka and Somero (1973) suggested that seasonal rate compensatory responses are due to fundamental changes in cellular chemistry.

3) Immediate rate compensation - Some aquatic ectotherms, mainly intertidal organisms, experience significant temperature changes on a diurnal basis and yet exhibit no unusual changes in metabolism (i.e. approximately a Q_{10} of unity). For example, Somero (1969) worked with pyruvate kinase in the Alaska king crab, an organism that experiences fluctuations within a range of 4°-$12^{\circ}C$ in its environment. He found two variants of the enzyme and concluded that both variants are formed by a temperature-dependent conversion of one protein

species. Newell (1966), working with an intertidal species of cockle and citing work with other intertidal organisms such as anemones, polychaetes, winkles and barnacles, concluded that the metabolism of these organisms is largely independent of rapid fluctuations in temperature (Q_{10} of approximately 1.2-1.3). The range for this temperature independence depends on the range of temperature to which the particular organism was subjected. Hazel and Prosser (1974), citing the same articles as Newell (1966) plus one that utilized a species of mussel, pointed out that although active metabolism was temperature dependent, the standard or resting metabolism was temperature independent. The range of temperature independence was again associated with the range of environmental temperatures. Hazel and Prosser (1974) also noted other authors who found similar evidence of immediate rate compensation in such organisms as amphipods, copepods, crayfish, oysters, garter snakes, salamanders, frogs, desert spiders, shore crabs, desert locusts and blowflies. It should also be noted that this phenomenon applies to tissue preparations as well.

Although much work in this area has been done with fish and intertidal organisms, little if any has been attempted with aquatic insects. In addition, we could find no evidence that anyone has attempted to relate these basic metabolic responses to temperature with the toxicity of pesticides in aquatic insects.

Parathion and Organophosphate Insecticides

After World War II, there was a large expansion in the number of insecticides available, due mainly to war-time research. These new compounds were mainly organic in nature. Many were chlorinated organic compounds such as DDT and chlordane but in recent years, many of these compounds have been banned due to findings that they were environmentally persistent, biologically accumulative, highly toxic and carcinogenic.

As a result, another group of insecticides, organophosphates (also developed during the war), have been used more extensively. This is the largest and most diverse group of insecticides in use today (McEwen and Stephenson, 1979). These insecticides are esters of alcohols and phosphoric acid or derivatives of phosphoric acid (O'Brien, 1967).

Parathion was discovered by Schrader in 1944. Chemically, this compound is defined as o, o-diethyl o-nitrophenyl phosphorothioate. It is one of the most toxic and widely used insecticides in North America, is broad spectrum in application to pest problems, is not bioaccumulative and is metabolized rapidly by most organisms. It is not highly soluble in water (1-24 ppm) and degrades rapidly in aquatic systems. Therefore, it is rarely found in water or sediment samples.

Mode of Action: Inhibition of Acetylcholinesterase

Early in these developments, it was recognized that organophosphates were inhibitors of acetylcholinesterase, resulting in a build-up of the neurotransmitter acetycholine at nerve synapses (Calhoun, 1959; McEwen and Stephenson, 1979). Although it was noted that phosphorothioates were not such potent inhibitors of acetylcholinesterase as their phosphate analogs, it was noted by Metcalf and March (1949) that there was little difference in toxicity between thiono forms and their oxygen analogs. Chamberlain and Hoskins (1951) also noted that thiophosphates were slower in toxic action but ultimately just as toxic as the phosphate analogs. They also observed that parathion was a poor acetylcholinesterase inhibitor in vitro and that toxic effects did not appear in cockroaches until 85% inhibition of acetylcholinesterase had occurred. They postulated that parathion was metabolically changed to paraoxon. Diggle and Gage (1951) found that parathion was converted to paraoxon in the liver and other tissues of rats, noting also that previous studies claiming inhibition of the enzyme by parathion was the result of impurities and that parathion is a poor inhibitor of cholinesterases. Metcalf and March (1953) made the

first observation of parathion conversion to paraoxon in insects by chromatographic methods. O'Brien (1960) stated that the activation of parathion to paraoxon may result in a 10,000-fold increase in inhibitory capacity. Metcalf and March (1949) found that paraoxon worked more rapidly and was more toxic to roaches than parathion. They also noted that the degree of cholinesterase inhibition correlated with the progression of toxic symptoms and that inhibition levels of greater than 90% were necessary for knockdown and death. Bigley (1966) confirmed these same observations with houseflies. In addition, he also found paraoxon to be approximatley 100 times more soluble in water than parathion. Dortland (1978) and Goodman (1979) also found decreased enzyme activity with increased concentration of parathion.

Paraoxon has a high affinity for acetylcholinesterase and rapidly phosphorylates the enzyme. After a few hours, the enzyme-inhibitor complex becomes permanent and therefore paraoxon and most organophosphates are considered to be irreversible inhibitors. Regeneration of the enzyme is the only means to regain full enzyme activity. Mengle and O'Brien (1960) found that cholinesterase inhibition was rapid and that houseflies recovered 90% of their enzyme activity within one day, however, there was no recovery of inhibition in in vitro studies. Brady and Sternburg (1966) found slow in vivo recovery of cholinesterase activity and attributed it to regeneration of the enzyme. Verma et al (1979) found that certain fish required up to seven days to recover full cholinesterase activity. They felt that, although some organophosphate poisonings may not result in their dealth, fish may be subject to predation before the enzyme activity could be regenerated. Symons and Metcalf (1978) found that the aquatic insect, Brachycentrus numerosus, exposed to fenitrothion for 24 hours, required up to 19 days to recover to normal activity. More importantly, affected organisms left the protection of their cases which also may permit predation.

The role of mixed-function oxidases in organosphosphate poisoning

It was established early in the development of organophosphate insecticides that phosphorothioates were converted enzymatically by various tissues in mammals and

insects to the more toxic phosphate form by oxidative (desulfurization) reactions (Diggle et al, 1951; Metcalf and March, 1953 and O'Brien, 1967). This reaction as well as hydroxylation of aromatic and alicyclic rings, hydroxylation of aliphatic side chains, dealkylation of aromatic and cyclic ethers, dealkylation of substituted amines, epoxidation of double bonds and oxidation of thioethers were associated with microsomal cell preparations in insects, similar to preparations from mammalian liver (Wilkinson and Brattsten, 1973). This wide ranging, non-specific enzyme system came to be known as mixed-function oxidase (MFO). Most of these reactions act to detoxify xenobiotics, but in the case of some insecticides, particularly thionophosphates, these enzymes actually activate the compound.

Kok and Walop (1954) found most of the oxidative activity in American cockroaches to be in the fat body. Hodgson and Plapp (1970) found oxidation of xenobiotics in houseflies to be dependent on a microsomal electron transport system which included NADPH and a carbon monoxide binding pigment similar to cytochrome P-450 in mammalian liver. They also found that the microsomal preparation from flies was similar in appearance to mammalian liver preparations under the electron microscope. Brattsten and Metcalf (1973) working with several species of flies found the MFO system to be responsible for the biochemical transformation of several insecticides.

Specific inhibitors of the MFO system have been discovered that advanced our knowledge on the toxicological implications of oxidative metabolism. MFO inhibitors may be used to increase the toxicity (synergize) of some insecticides (i.e. pyrethrins) or, in the case of activation reactions, cause a decrease (antagonize) in the toxicity of phosphorothioates. Nakatsugawa and Dahm (1965) reported that several synergists, particularly piperonyl butoxide (PBO), could inhibit MFO activity and, therefore, prevent the activation of parathion. Philleo et al (1965) working with methylenedioxyphenyl compounds as inhibitors of the hydroxylation of naphthalene in

houseflies, found that 14 of these compounds (including PBO) would inhibit this reaction. Terriere (1968) also noted that the conversation of parathion to paraoxon was inhibited by PBO. Casida (1970) found that methylenedioxyphenyl compounds enhanced the insecticidal properties of certain chemicals by inhibiting the MFO system in microsomes. Brattsten and Metcalf (1973) also found that MFO activity could be inhibited by PBO. Estenik and Collins (1979) concluded that the MFO system in Chironomus riparius was highly active and could be completely inhibited by PBO.

The activity of the MFO system can therefore be approximated in many insects by the ability of PBO to inhibit the conversion of parathion to paraoxon. This conversion would be reflected in the interactive toxicity ratio (EC_{50} parathion + PBO/EC_{50} parathion). A high ratio would indicate higher MFO activity. This method was also used by Estenik and Collins (1979).

Toxicity studies with aquatic insects

Although some toxicity work has been conducted with aquatic insects and insecticides, very little has been done relating toxicity to temperature.

Jensen and Gaufin (1964) worked with two species of stonefly naiads and several organic pesticides. All experiments were conducted at approximately 13°C and they established lethal levels as well as effects on molting. Sanders and Cope (1968), also working with stonefly naiads (3 species) and pesticides, measured only the acute toxicity at 15.5°C. They found the smallest species and the smallest members of each species to be the most susceptible. Mulla and Khasawinah (1969) studied the problem of midge populations as pests in sewage oxidation ponds. They evaluated the effectiveness of several insecticides, including parathion, at 78°F in the field and laboratory. This research was followed by field testing of seven organophosphorus insecticides against midge larvae in flood control channels (Ali and Mulla, 1976). Federle and Collins (1976) conducted studies with three species of pond insects. They used representatives of four classes of insecticides (organochlorines,

organophosphates, carbamates and pyrethrins). All tests were conducted at 25°C and only acute toxicity was measured. Symons and Metcalf (1978) studied the effects of fenitrothion to the caddis fly larvae Brachycentrus numerosus. They ran all tests at 5°C. They measured acute toxicity as well as behavior in cases. They found that all the organisms that left their cases after a 24-hour exposure were dead within 30 days. They also discovered that survivors recovered completely in 19 days.

Several other studies have been conducted with aquatic insects and insecticides, but we have been unable to locate any reports where temperature was varied to determine its affects on toxicity from either a seasonal or diurnal basis. However, one study conducted on the terrestrial cabbage looper found that a reduction in temperature caused a reduction in toxicity. This study was conducted from an agricultural control standpoint and acclimation of the organisms was not done. A laboratory colony was used and tests were run at 32°C and 10°C. Decreases in toxicity with temperature varied between insecticides used (Chalfont, 1973).

Project Objectives

Six species of immature aquatic insects representing four orders (Plecoptera, Ephemeroptera, Trichoptera and Diptera) were collected in their natural habit in summer and winter seasons for experimentation. Laboratory experiments were conducted with these insects at temperatures of 4°C and 22°C which approximated the seasonal temperatures of the streams in which they were collected. A laboratory culture of the seventh species (Chironomus) was acclimated to comparable temperatures before tests were conducted.

The conceptual objectives of this project were:

1. To determine if winter and summer forms of aquatic insects differ significantly in their susceptibility to parathion and paraoxon at temperatures equivalent to their natural habitat.
2. To determine the relative toxicity of parathion and paraoxon to aquatic insects at high (22°C) and low (4°C) temperatures.
3. To determine if, in aquatic insects, oxidative metabolism affects the toxicity of a phosphorothioate insecticide (parathion) and its oxygen analogue (paraoxon).
4. To determine if acetylcholinesterase transformations occur in aquatic insects as a response to seasonal changes.
5. To determine the threshold of in vivo acetylcholinesterase inhibition associated with observable symptoms of toxicity of parathion in Chaoborus.
6. To determine the stability of inhibited acetylcholinesterase in Chaoborus exposed to parathion as a means of estimating the potential for cumulative acetylcholinesterase inhibition in insects exposed intermittently in streams.

The experimental objectives of this project were:

1. To collect six species of aquatic insects from their natural habitat in the summer and winter for laboratory experimentation, adding a seventh species which was reared in the laboratory (Table I provides the genera, orders and characteristics of the aquatic insects utilized in the project).
2. To measure the average wet weight, dry weight, percent water content and lipid content of the insects.
3. To measure the respiratory rate (oxygen consumption) of winter and summer forms of aquatic insects at 4°C and 22°C, respectively.
4. To measure the acute toxicity (48-hour LC_{50}) of parathion and paraoxon (with and without piperonyl butoxide, a mixed-function oxidase inhibitor) in winter and summer forms of aquatic insects at 4°C and 22°C, respectively.
5. To measure the acute toxicity (48-hour LC_{50}) of parathion to the winter form of Chaoborus and the laboratory culture of Chironomus at 22°C and 4°C, with and without piperonyl butoxide.
6. To establish the optimum temperature and substrate concentration conditions for in vitro acetylcholinesterase activity in the aquatic insects, and to measure the in vitro acetylcholinesterase activity in winter and summer forms at 4°C and 22°C, respectively.
7. To measure the in vitro paraoxon inhibition (I_{50}, molar) of acetylcholinesterase in summer and winter forms of aquatic insects at both temperatures, 4°C and 22°C.
8. To measure the rate of inhibition of acetylcholinesterase in Chaoborus exposed to parathion and to monitor the regeneration of free enzyme from inhibited enzyme in Chaoborus held in clean water.

Table 1

Characteristics of Experimental Insects

Species	Order	Common Name	Habitat	Habits
Allocapnia sp.	Plecoptera	Stonefly	Stream (riffles)	- Leaf Packs - Detritus Feeder
Stenonema femoratum	Ephemeroptera	Mayfly	Stream (riffles)	- Rock Clinger - Scraper
Stenonema vicarium	Ephemeroptera	Mayfly	Stream (riffles)	- Rock Clinger - Scraper
Cheumatopsyche sp.	Trichoptera	Caddisfly	Stream (riffles)	- Case Builder - Net Spinner
Hydropsyche sp.	Trichoptera	Caddisfly	Stream (riffles)	- Case Builder - Net Spinner
Chaoborus sp.	Diptera	Phantom Midge	Lakes and ponds	- Pelagic - Predator
[a]*Chironomus riparius*	Diptera	True Midge	Streams	- Burrower - Detritus Feeder

[a]Laboratory Colony

Methods and Materials

Collection sites, Collection methods and Holding Conditions in the Laboratory

Seven species of immature aquatic insects were used in these experiments. They were selected because of their availability in large numbers in both winter and summer seasons and their ease of maintenance in the laboratory.

Three species of immature stream insects (Allocapnia sp., Stenonema femoratum and Stenonema vicarium) were collected from Hayden Run, a small stream fifteen kilometers north of the OSU campus. All collections were made between its confluence with the western side of the Scioto River and a ten meter waterfall approximately 300 meters upstream. Groundwater contributes a portion of the stream flow in this section and, therefore, it remained open during the winter. The stream is approximately two to three meters wide with a mean depth of 20 centimeters. The substrate is rocky and contains very little silt. The upper reaches of the stream pass through pasture land and remote suburban areas while the stretch below the falls flowed through a heavily shaded ravine. Stream temperatures measured randomly during collections never exceeded 22°C in the summer and were not less than 1°C in the winter.

Two additional species (Cheumatopsyche sp. and Hydropsyche sp). were collected from the Scioto River 200 meters below the dam at Griggs Reservoir five kilometers west of OSU. The stream, approximately 20 meters wide at this point, has a mean depth of 50 centimeters but this factor varied, depending on water releases from the reservoir. The water was more turbid and contained more silt than Hayden Run. The substrate was rocky. Winter stream collection temperatures varied from 0°C to 8°C but the river remained open at this point due to rapid water movement. Summer collection temperatures varied from 18°C to 27°C.

Collections from both streams in the summer were obtained by handpicking desirable specimens from rocks. Buckets containing the specimens were kept cool by

immersing them in the stream. Summer collections at both sites were made from mid-June through September.The insects were immediately transported to the laboratory where they were segregated according to species into shallow trays of water and maintained in incubators at 22°C with aeration and 15 hours of light. Insects were utilized for experiments within two days of collection.

Winter collections were made from mid-December through March. Due to the large accumulations of tree leaves among the rocks and also the rigors of collecting at cold temperatures, collections were made by placing a square frame dip net downstream of the collection point and dislodging the insects from the rocks with small brushes. Insects and debris, swept into the net by the stream flow, were placed into collection buckets containing stream water and transported to the laboratory. There, the insects were sorted from the debris by hand and kept at 1°C during this process by placing the sorting trays on ice. Sorted collections were maintained at 4°C in incubators with aeration and a ten-hour photophase. Insects were utilized for experiments within two days of collection.

Laboratory tap water used for maintenance of the insects in the lab as well as for the experiments was collected 24 hours in advance of its use. The source of this water (Columbus City System) is the Scioto River and Big Walnut Creek. This water is softened and chlorinated by the city treatment plants. All tap water used in these experiments was filtered through activated charcoal using Tygon tubing as connectors. The filter, constructed of a poly-vinylchloride tube 1 meter long and 10 centimeters in diameter contained high grade, fine granule carbon (Barnebey-Cheney coconut shell base AC, 6 x 10 Tyler screen). The column was mounted vertically and the flow was against gravity. This treatment effectively removed chlorine (Shank, thesis 1976), trace organics (Shank and Collins, unpublished) and some metals (Shank and Collins, unpublished).The water was then aerated for 24 hours in Nalgene carbouys placed in incubators at the appropriate temperature (4°C or 22°C).

Chaoborus sp., was collected from three cement-lined ponds at the U.S. E.P.A., Toxicology Station in Newtown, Ohio. Numerous other aquatic insects were present in these ponds. Chaoborus, a pelagic species, was collected with fine mesh dip nets, placed in buckets containing the pond water and transported to Columbus. During the two hour drive, temperature was maintained in the collection buckets by adding ice. Pond temperatures varied from 18°C to 30°C in the summer and 0°C to 8°C during the winter collection period. These insects were maintained in deep plastic trays placed in incubators at 22°C (15 hours of light) in the summer and 4°C (10 hours of light) in the winter. Light in each incubator was supplied by one 8 watt fluorescent tube. With minimal aeration Chaoborus could be maintained under these conditions for more than two weeks in the laboratory with no observable stress. Quantities of Daphnia sp. were collected from the same ponds to provide food for Chaoborus during the holding period in the laboratory. The collected Daphnia were maintained in the same container with the Chaoborus.

Chironomus riparius was the only species that was cultured in the laboratory. This particular colony had been reared in the laboratory for nine years prior to these experiments. A starter colony was originally collected below a city sewage treatment facility on the Scioto River downstream from Columbus (Estenik thesis). They were reared in 80 liter all-glass aquaria with screen flight cages (60 cm long, 30 cm wide and 45 cm high) mounted over the tanks to provide a swarming and mating space for the adults. The larvae were fed a fine grained commercial trout chow (Purina) three times a week. The substrate in which they constructed burrows was formed by larval excrement, organic matter derived from unconsumed food, etc. The midges were maintained continuously at room temperature (20°C-25°C) with a 14 hour photophase. Larvae used for "summer condition" experiments were removed directly from the aquaria. Acclimation of larvae to simulated winter conditions was accomplished by placing several thousand organisms with substrate from the cultures into deep aerated

trays in the incubator at 22°C and 14 hours of light per day. Over a 30 day period the temperature was gradually decreased (approximately 5°C per week) to 4°C and the light period shortened to 10 hours. The insects were then used for "winter condition" experiments.

Experimental Organisms

General ecological data on the insects used in these experiments are found in Table 1. Allocapnia sp.* was the only representative of the Stoneflies (Plecoptera) and was only collectable in the winter season. These organisms are detritivores inhabiting coarse sediments, debris jams and leaf packs in streams (Merritt and Cummins, 1978). They were found in leaf packs throughout the stream from December through March but were most abundant in January and February. Emergence and mating occurred during the latter months and adults were observed crawling on the snow in great numbers. The numbers of nymphs and adults declined drastically in March, and were not found from April through November. Therefore, only winter nymphs were used in these experiments.

A search of the literature revealed that finding only winter forms of Allocapnia would not be unusual. Khoo (1968) reported that Capnia bifrons, a winter species closely related to Allocapnia, diapaused in the substratum during the warm months. Moreover, Harper and Hynes (1970), working with seven species of winter stoneflies including three species of Allocapnia, found that they diapaused as early instar nymphs deep in the gravel of the stream bottom during the warmer months. This pattern was verified by Coleman and Hynes (1970, 1971). Our observations in the laboratory indicated that this species could be kept at warmer temperatures (10°C) for no more than a few hours.

*Probably Allocapnia vivapara (Claassen) due to the absence of wings in the adult males (Usinger, 1956; Mike Glorioso, personal communication).

Last nymphal instars of two species of mayflies used in the experiments were both of the genus Stenonema (S. femoratum and S. vicarium). They are common stream species which cling to the rocks, scraping algae and organic debris from the rock surfaces (Merritt and Cummins, 1978). These two species, predominant among numerous species of mayflies living in the stream, were found in riffle areas of Hayden Run. S. vicarium, most abundant as large nymphs during the winter, appeared to have one major adult emergence per year during May and June. After this late spring emergence it was difficult to find large nymphal instars. Coleman and Hynes (1970) noted that S. vicarium in a small Ontario stream appeared to have early egg hatches and that nymphs grew steadily throughout the year. S. femoratum was the most abundant species of mayfly at Hayden Run during the summer months and large instars were present all through the year. This species appeared to have several distinct cycles of emergence throughout the year. Such an emergence pattern would explain the abundance of large instars of S. femoratum at Hayden Run over a longer period of time.

The two species of caddisflies (Trichoptera) used in the experiments, both in the family Hydropsychidae, are filter feeders. The two species, Hydropsyche sp. and Cheumatopsyche sp., constructed nets at the open end of their tubes which were attached to the bottoms and sides of rocks.

Muller (1956) and Chutter (1963) noted that filter feeding organisms were generally present in dense populations below lake outlets, postulating that this was due to the large plankton populations in the lakes. In addition, Chutter (1963) concluded that impoundments allow abrasive material such as sand to settle out before water is discharged. Accordingly, large populations of these organisms were formed 200 meters below the outfall from Griggs Reservoir in Columbus. Evidently, these species have staggered cycles of emergence because large instars could be collected throughout the year. Adult emergence occurred throughout the summer with the peak in the spring. Hynes (1970) concluded that all species of Trichoptera are univoltine.

Two species of flies (Diptera) were used in the experiments. Chaoborus sp. is pelagic and predatory, feeding on the many small crustaceans present in their environment. We found that they emerged from April through September with a peak in June at the Newtown, Ohio location. Large instars became scarce after the June peak emergence. However, during the warm summer months these organisms go through a complete life cycle in 30 days. Therefore, by mid-summer they were abundant once more.

The other Dipteran, Chironomus riparius, was reared in the laboratory. This species burrows in the soft substrate and is a detritus feeder. They complete a life cycle in 30 days under laboratory conditions. Large instars were always available for experiments.

Physical Characteristics of Experimental Insects

Average wet weight (mg/insect) was determined for both the winter and summer forms of each species. These data were acquired from weights taken during the course of the other experiments and analyses (respiration, lipids, percent water and acetylcholinesterase activity). Weight measurements were obtained by counting out from 3 to 100 insects, depending on the species and experiment, drying them briefly on a paper towel and then weighing them on a Mettler analytical balance. Generally each species from a given season was fairly uniform in size, particularly because of the selection process used by the investigators. Variance and standard deviation of mean values were not calculated.

The average percent water of each organism was determined only once. Six to fifty organisms, depending on species and size, were weighed as previously described in a tared aluminum foil tray which had been dried in an oven at 80°C. The insects and tray were then placed in a drying oven at 80°C for 24 hours. The tray and dried organisms were then cooled and reweighed. Loss of weight from the insects was assumed to be water.

Average percent lipid of each species was determined by modifying the method developed by Bligh and Dyer (1959) to accommodate a smaller mass of organisms than the original technique. The procedure involves homogenization of the sample with a Virtis homogenizer in distilled water, methanol and chloroform. Using cod liver oil as a standard, 97% recovery of lipids was obtained. Approximately one gram of insects, weighed as previously described, was used in each trial. Duplicate weighings were performed but variance and standard deviation of mean values were not determined.

Metabolic Rate

Respiration rates were determined as ul oxygen/mg organism/hour using a Warburg respirometer. This standard manometric technique is described in Umbreit, 1964. Temperature was maintained within $\pm$ 1^{o}C by submerging flasks containing the insects into a recirculating water bath. Thermo-barometer readings (flasks without the insects) were used to adjust for effects of changes in barometric pressure or temperature on experimental values. The glass flasks were approximately 20 ml in size and contained a small glass well in the center of the flask. Ten percent potassium hydroxide (0.2 ml), placed into the center well with a 2.5 cm^{2}fluted wick made from Watman #1 filter paper, absorbed any carbon dioxide given off by the organisms during the experiment. Preliminary experiments were conducted under light and dark conditions, with and without substrate, to determine the minimum respiratory rate of each species. Light conditions had little effect on respiratory rate, but the presence of substrate (1 cm by 2 cm strips of paper toweling) was needed to promote quiescence and reduce fluctuations in oxygen consumption of the clinging and burrowing insects. All of the species except Chaoborus sp. hid under or clinged to these strips.

Three to eight insects, depending upon size, were placed in the flask with four ml of carbon-filtered water. The flasks remained immersed in the water bath for fifteen minutes to allow the temperature in the flask to equilibrate and permit the insects to adjust to their new environment. Each experiment ran for 2 to 6 hours depending on

the temperature and species. The insects were weighed at the end of each experiment.

These experiments were conducted at 22°C with insects collected in the summer and 4°C with those collected in the winter. In addition, tests were conducted at 4°C on summer organisms and 22°C on winter organisms after a 24 hour acclimation period. Acclimation was accomplished by placing trays of organisms in an incubator at the temperature to which they were naturally acclimatized (4°C in the winter and 22°C in the summer). The incubator was then set at the alternate temperature and allowed to warm or cool for 24 hours. Experiments were conducted at the resulting temperature.

Three replicate flasks were tested during each experiment and each experiment was repeated two to three times. Mean and standard deviation values were determined for all experiments.

Toxicity Tests

These tests were conducted following guidelines established in USEPA publications and standard methods (USEPA, 1975 & 1978, APHA, 1980).

Tests were conducted using 500 ml of water in 1 liter beakers. Filtered, aerated tap water was prepared as previously described. Stock solutions of test chemicals were prepared from high purity stock and dissolved in reagent grade acetone. The acetone solutions were added in 0.5 ml quantities to the 500 ml of test water to achieve final test concentrations. Controls containing water only or water with 0.5 ml of acetone were utilized for all experiments. All experiments were conducted for 48 hours in incubators at designated temperatures and light cycles. Fiberglass screen substrates (3 cm square) were utilized in each beaker for the clinging and burrowing species.

Because of variations in morphology and habits among the organisms, differing end points were used to measure the toxic effects of chemicals for each species.

However, all end points were based on the ability of the organisms to maintain normal equilibrium or posture. At the end of an experiment the organisms and water were poured into shallow trays for observations on toxic effects . Different numbers of tests were conducted on each seasonal form of each species depending on their abundance at that time. Data from all similarly designed experiments were pooled and hand plots (% mortality vs. concentration) were made on standard log-probit paper. EC 50 values were interpolated from these plots. Tests were conducted using parathion and paraoxon, each with and without piperonyl butoxide (PBO). Animals were pretreated with 0.5 mg/l of PBO for four hours prior to addition of the insecticides. The concentration of PBO was maintained at 0.5 mg/l during the course of the experiment and an additional control containing 0.5 mg/l of PBO was utilized.

Summer forms of each species were tested at 22°C and winter forms at 4°C. In addition, winter forms of Chaoborus sp. and both warm and cold acclimated forms of C. riparius were acclimated over 24 hours to the alternate temperature as previously described. They were then tested at this temperature.

In vitro Acetylcholinesterase Activity

Several different tests were conducted to measure the in vitro acetylcholinesterase activity of each species. The assay utilized for all tests was a photometric technique developed by Ellman, et al (1961), employing an artificial substrate, acetylthiocholine (ATCH) and a trapping agent, dithiobisnitrobenzoate ion (DTNB). The Ellman technique is based on the following reactions:

$$\text{ATCH} \xrightarrow{\text{acetylcholinesterase}} \text{thiocholine and acetate}$$

$$\text{thiocholine} + \text{DTNB} \longrightarrow \text{yellow color}$$

The acetylcholinesterase data obtained with the artificial substrate, acetylthiocholine are similar to those obtained with the natural substrate,

acetylcholine (Ellman, et al., 1961). Riddles, et al. (1979) confirmed this test and established the molar absorption coefficient of the TNB dianion as 14,150 at 412 nm. The actual color is produced by the TNB dianion which is formed when a thiol group reacts with DTNB. The other product of this reaction is a mixed disulfide. However, DTNB reacts with any free thiol group, including endogenous thiols in homogenates. Analytical artifacts due to endogenous thiols were eliminated by mixing excess DTNB with the enzyme homogenate 1 hour prior to use. Any free thiol groups present (i.e. cysteine) would react with the DTNB prior to addition of the substrate and any endogenous chromogen so produced could be accounted for with a control (homogenate without the substate).

Enzyme homogenates were prepared with whole insects that had been frozen prior to the experiment. Insects were homogenized in 0.05 M phosphate buffer (pH 7.5) and 0.05 M sodium chloride with a Virtis homogenizer in a beaker immersed in ice. Quantity of buffer varied with the species used. The homogenate was then centrifuged at 2000 xg in a refrigerated centrifuge for 20 minutes. The supernatant was decanted, mixed with an excess of DTNB (0.4 ml/ml homogenate), then held at room temperature for one hour prior to use. It was recooled before using. The slight warming during this period accelerated the reaction of the endogenous chemicals containing thiol groups with DTNB and did not harm the enzyme (Shank and Collins, unpublished).

The DTNB solution was prepared by mixing the powdered DTNB with pH 7.0, 0.1m phosphate buffer and a small amount of sodium bicarbonate. Substrate solutions were prepared by dissolving crystallized ATCH in distilled water. The number of insects used to prepare the homogenate varied among species in order to achieve a measurable level of acetylcholinesterase activity.

The pH and ionic strength of the Tris buffer were held at 8.0 and 0.1 M, respectively, for all preparations. These values were based on other work conducted in our laboratory on C. riparius acetylcholinesterase (Randall Detra, personal

communication) as well as the general literature. Although the optimum temperature for the acetylcholinesterase preparations of each species was determined, most experiments were conducted at nominal summer (22°C) and winter (4°C) temperatures. The optimal substrate concentration for each form of each species was determined at both 4°C and 22°C using a Beckman DU spectrophotometer and chart recorder. Results from these experiments were converted to umoles ATCH/organism/minute and analyzed graphically by plotting enzyme activity versus temperature or substrate concentration.

In vitro Inhibition of Acetylcholinesterase

Inhibition experiments were conducted by preparing homogenates as described previously and using the same conditions and assay method (Ellman technique). However, in the inhibition experiments the enzyme homogenate was incubated with buffer and inhibitor (parathion or paraoxon) for 1 minute prior to assay. Various *in vitro* concentrations of the inhibitors were prepared by adding 0.1 ml of acetone solutions of chemical to the homogenate. An acetone control was used in each experiment. Results were plotted on log-probit paper, percent inhibition versus molar concentration of the inhibitor and I_{50} values were determined for each form (summer and winter) of each species at 4°C and 22°C.

In vivo Inhibition of Acetylcholinesterase and Regeneration of Inhibited Enzyme

In vivo inhibition experiments were conducted with the summer form of *Chaoborus sp.* using parathion. Twenty to thirty organisms were placed in 500 ml of carbon filtered water in 1 liter beakers and exposed to either 10 ug/l of parathion for 5.25 hours or 1 ug/l for 48 hours. Acetone controls were run concurrently. At specific time periods during exposure, all organisms were removed from one beaker, rinsed in distilled water and frozen. The frozen samples were homogenized in ATCH solution using the protective substrate technique (Van Asperen, 1958 & 1960) and immediately assayed spectrophotometrically. Less than 45 seconds elapsed between mixing with

ATCH until the assay was started. Percent inhibition of the enzyme was determined by comparison to the acetone controls.

Experiments were also conducted on the summer form of Chaoborus sp. to determine the regeneration rate of acetylcholinesterase activity after exposure to an inhibitor. Approximately 300 organisms were exposed to 1 ug/l of parathion at 22°C for 24 hours in 2 liters of carbon filtered water. An identical exposure was conducted with acetone as a control. At the end of the exposure period dead and affected animals were counted. Those animals appearing normal were sorted into smaller groups of 10 to 15 and placed into 1 liter beakers containing 500 ml of carbon filtered water. One group of organisms from each exposure was selected at this time and assayed for acetylcholinesterase activity. Thereafter, at 24 hour intervals, two beakers from each group of exposed animals were removed, rinsed in clean water, weighed and frozen for acetylcholinesterase assay. Dead and affected animals were counted and removed. The experiments lasted 7 days. Two such experiments were done. Percent inhibition of the enzyme was determined by comparing the activity of the organisms exposed to parathion with the acetone controls.

Results and Discussion

Emergence Patterns and Seasonal Effects on Size

Weight differences between winter and summer forms of insects varied with each species (Table 2). Generally, compensation for size differences between seasonal forms was accomplished by careful selection of uniform specimens on the part of the investigator.

The species with short, defined emergence periods in the spring had larger instars present during the winter. Those having a longer emergence period (i.e. emerged sporadically throughout spring and summer) exhibited no difference in body sizes between winter and summer forms. However, the two Trichopterans, Chematopsyche sp. and Hydropsyche sp., had a distinct emergence period in the spring and also appeared to have a life cycle longer than one year. As a result, they were available throughout the year, but the summer forms were distinctly smaller than the winter forms. Chaoborus sp. emerged throughout the warm months with a peak emergence in early spring. During the summer a complete life cycle required 30 days or less. They were therefore available throughout the year but were slightly larger in the winter. The most restrictive emergence pattern, other than Allocapnia sp. which was not available in the summer, occurred with S. vicarium, which emerged entirely in late May and early June. After this emergence only the tiniest instars were available until very late in the summer. S. femoratum emerged throughout the warm months and was available in large instars at those times but all emergences ended by early autumn. Water temperatures remained high enough for continued growth of all species into late autumn and some species apparently continued to grow throughout the winter. Consequently, large instars were available during the winter and they would be the first forms to emerge with the advent of warm weather in the spring.

Water Content

Considerable variation existed in water content (range of 66-93%) among the various species (Table 2). Generally the Dipterans had the highest water content (83%

Table 2

Average Wet Weight, Water Content
and Lipid Content of Experimental Animals

	Avg. Wet Wt. mg/Insect		Avg. % H_2O	Avg. % Lipids
	Summer	Winter		
Allocapnia sp.	-	5	-	5.8
S. femoratum	20	20	77	3.0
S. vicarium	20	22	79	3.5
Cheumatopsyche sp.	9	14	66	9.6
Hydropsyche sp.	18	27	68	6.2
Chaoborus sp.	4	6	93	1.5
[a]*C. riparius*	6	6[b]	83	1.6

[a]Laboratory culture

[b]Acclimated to winter conditions in laboratory.

and 93%), the mayflies had the second highest (77% and 79%) and the Trichopterans had the lowest (66% and 68%). The Dipterans were worm-like in appearance with thin integuments that were easily damaged. Although the Trichopterans also were worm-like, they had very thick, tough integuments that were not easily punctured. The mayflies were quite chitinous and had hard integuments.

Lipid Content

The Trichopterans also had the highest lipid content (6.2 to 9.6%) of the species examined and the Dipterans had the lowest (1.5to 1.6%) (Table 2). The mayflies and stonefly fell in between (3.0 to 5.8%). An inverse trend between body water content and lipid content was evident, i.e. those insects with the most body water had the least fat and vice-versa. No trends were noted among body size and lipid content.

Respiration Rates

The winter form of Allocapnia sp. had the highest respiration rates among all insects at 4°C and 22°C (Table 3). Summer forms of this species were not available. Because winter was its only active period, one might expect above average respiration rates in Allocapnia at 4°C (winter form). This species exceeded that expectation because the respiration rate of Allocapnia at 4°C corresponded closely to the 22°C respiration rates of the summer forms of other species. Moreover, when Allocapnia was acclimated overnight to 22°C, the respiration rate at 22°C was more than double the 4°C rate and was significantly higher than 22°C respiration rates of other species. Clearly, Allocapnia consumed more oxygen at any temperature than the other species, a characteristic which may be related to its unusual life cycle and ecological niche.

Chaoborus sp. had the lowest respiration rate among summer and winter forms at both temperatures. This insect remained motionless in water and twitches only occasionally to change positions. It was the least active of all the species tested.

For all species except S. vicarium, respiration rates increased nearly 5-fold, comparing winter forms at 4°C with summer forms at 22°C. S. vicarium (3.3-fold) had

Table 3

Respiration Rates (O_2 Consumption) of Experimental Insects at Two Temperatures, ul O_2/mg/hr ($\pm$ S.D.)

	Winter Form				Summer Form			
	4°C		22°C		4°C		22°C	
Allocapnia sp.	0.26	(.04)	0.61	(.09)	-		-	
S. femoratum	0.065	(.003)	0.39	(.02)	0.07	(.01)	0.34	(.08)
S. vicarium	0.10	(.04)	0.49	(.08)	0.06	(.01)	0.33	(.08)
Cheumatopsyche sp.	0.13	(.02)	0.35	(.04)	0.08	(.01)	0.54	(.06)
Hydropsyche sp.	0.09	(.01)	0.37	(.05)	0.07	(.01)	0.47	(.04)
Chaoborus sp.	0.06	(.01)	0.275	(.03)	0.02	(.01)	0.30	(.05)
*C. riparius**	0.085	(.03)	0.49	(.10)	0.03	(.01)	0.44	(.10)

*Laboratory Culture

one of the higher winter rates and lower summer rates. This was also the only species that was distinctly univoltine and had a short term, single emergence in the spring. Much of its growth occurred during the winter months.

Generally, at the same temperature, respiration rates of summer and winter forms were not strikingly different. Acclimation of a seasonal form from one temperature to another was readily accomplished without apparent adverse biological effects. This would indicate that most of these species are not suppressed by seasonal limitations. The exception was the summer form of the two Dipterans, Chaoborus sp. and Chironomus riparius. Although the winter forms of these species could be acclimated to 22°C and respiration rates were comparable to their summer form at 22°C, the summer forms acclimated to 4°C had respiration rates much lower than the winter forms of these species at 4°C. The rates were barely measurable on the equipment used and the organism showed little or no movement. This result would indicate that these species must undergo significant physiological changes when acclimatized from summer to winter conditions.

There were no strong trends between body size and respiration rate. These two factors appeared to vary independently when respiration rates were indexed on a mg body weight basis.

Toxicity Experiments

Results for toxicity tests with summer and winter forms are summarized in Table 4. Allocapnia sp., which had the highest winter respiration rate, was more sensitive to parathion and paraoxon than the winter forms of the other species. The winter EC50 values of this species were similar to the summer EC50 values for the other species and were much less than the winter EC50 values of the others.

The EC50 values for parathion were very similar among the summer forms of all species tested. This is also true for the winter forms, with the exception of Allocapnia sp., which is only found in the winter, and C. riparius, which was artificially acclimated to winter conditions.

Table 4

Toxicity Data of Experimental Insects (48 hour EC_{50}) for Parathion (PT), Paraoxon (PO), With and Without PBO ($_u$g/L)

		Summer Form[a]				Winter Form[b]			
		PT	PT + PBO	PO	PO + PBO	PT	PT + PBO	PO	PO + PBO
Allocapnia sp.		-	-	-	-	2.2	21.0	2.5	2.6
S. femoratum		1.7	2.4	4.5	4.9	30.0	46.0	32.0	36.0
S. vicarium		-	-	-	-	29.0	38.0	18.0	23.0
Cheumatopsyche sp.		2.5	2.7	14.5	13.5	21.0	39.0	67.0	62.0
Hydropsyche sp.		1.3	2.4	10.0	6.0	36.0	-	68.0	-
Chaoborus sp.	4°C	-	-	-	-	48.0	56.0	450.0	410.0
	22°C	1.0	5.9	22.0	26.0	0.8	4.2	21.0	-
C. riparius	4°C	-	18.0	-	11.5	8.4	31.0	12.0	12.5
	22°C	1.6	10.0	3.0	2.5	1.8	6	2	2

a. Tested at 22°C except as noted.

b. Tested at 4°C except as noted.

It should be noted that paraoxon is the active, toxic form of parathion by virtue of its stronger potency as an acetylcholinesterase inhibitor. Parathion is converted to paraoxon in most organisms by the mixed function oxidase system (Chamberlain et al 1951, Diggle et al 1951, Metcalf et al 1953a and 1953b, Kok et al 1954, Nakatsugawa et al 1965, O'Brien 1967, Terriere 1968, Hodgson et al 1970, Brattsten et al 1973). In experiments with terrestrial insects, paraoxon often may be more toxic than parathion (Metcalf et al 1949, O'Brien 1960 and Bigley 1966). However, in the summer forms of the insects used in this study, parathion was 2 to 22 times more toxic than paraoxon. There was little difference in the toxicity of parathion and paraoxon among the winter forms except with the Trichopterans, which were 2 to 3 times more sensitive to parathion than paraoxon, and Chaoborus sp., which was 9 times more sensitive to parathion.

Paraoxon is approximately 100 times more soluble in water than parathion due to its greater polarity (Bigley, 1966). This probably results in a decreased ability of paraoxon to penetrate the insect integument due to the non-polar component of the integument and the hydrophilic nature of the compound. In experiments with terrestrial insects the compound is applied directly to the organism by topical application and not exposed to an aqueous solution, such as in these experiment.

Experiments were conducted using piperonyl butoxide (PBO) in conjunction with parathion and paraoxon (Table 4). PBO acts as an inhibitor of the mixed function oxidase (MFO) system (Nakatsugawa et al 1965, Philleo et al 1965, Terriere 1968, Casida 1970 and Brattsten 1973) and, therefore, may antagonize the toxic effect of parathion by preventing the formation of paraoxon. The difference in toxicity between experiments with parathion alone and parathion with PBO is a good indicator of insect MFO activity or the utilization of MFO to metabolize a toxic compound.

The summer forms of the insects used in these experiments exhibited interactive toxicity ratios of less than 2 (EC_{50} parathion plus PBO/EC_{50} parathion), with the

exception of Chaoborus sp. and C. riparius. Chaoborus sp. exhibited a toxicity ratio of six and C. riparius, nine, which agrees with previous work by Estenik and Collins on the latter species (1979). This indicates that these two Dipteran species have a very active MFO system and rapidly metabolize parathion to paraoxon.

The response to treatments with parathion or parathion plus PBO in winter forms ($4^{o}C$) resulted in toxicity ratios that were similar to summer forms ($22^{o}C$) except that the ratio for Chaoborus sp. was near unity and that for C. riparius was reduced to approximately three. This was probably due to the greatly decreased respiration rates and reduced chemical activity associated with the cooler temperature.

However, Allocapnia sp. which maintains a high respiration rate and activity rate at low temperatures exhibited an interactive toxicity ratio of nine at low temperature, which is much greater than the other insects. This is also indicative of this organism's greater winter activity and high respiration rates, and suggests that Allocapnia has a highly active MFO system.

The EC_{50} values for paraoxon, with and without PBO, were very similar at the same temperature, indicating that oxidative metabolism is probably not critical in the metabolism of paraoxon.

Allocapnia sp. had the highest MFO activity (based on interactive toxicity ratios) and was also the most sensitive to parathion among the winter organisms in this study. The activation of parathion to paraoxon by the MFO system is critical to the toxic action of this chemical. An organism with very active oxidative metabolism would be expected to be very sensitive to parathion due to the activation process.

Chaoborus sp. was the most sensitive summer form and had the greatest MFO activity at $22^{o}C$ (highest interactive toxicity ratio). However, it was the least sensitive organism to parathion in the winter and MFO activity was severely reduced (interactive toxicity ratio of one). The higher temperatures in the summer coupled with increased respiration and increased oxidative metabolism make this organism vulnerable to parathion.

Experiments were also conducted with winter forms of C. riparius and Chaoborus sp. at 22°C and summer forms at 4°C by acclimation over a 24-hour period. Generally, data for 4°C in summer forms were similar to 4°C data in winter forms; 22°C data in the winter were similar to 22°C data in the summer. This would indicate that no real differences in toxicity exist between summer and winter forms except those dictated by temperature.

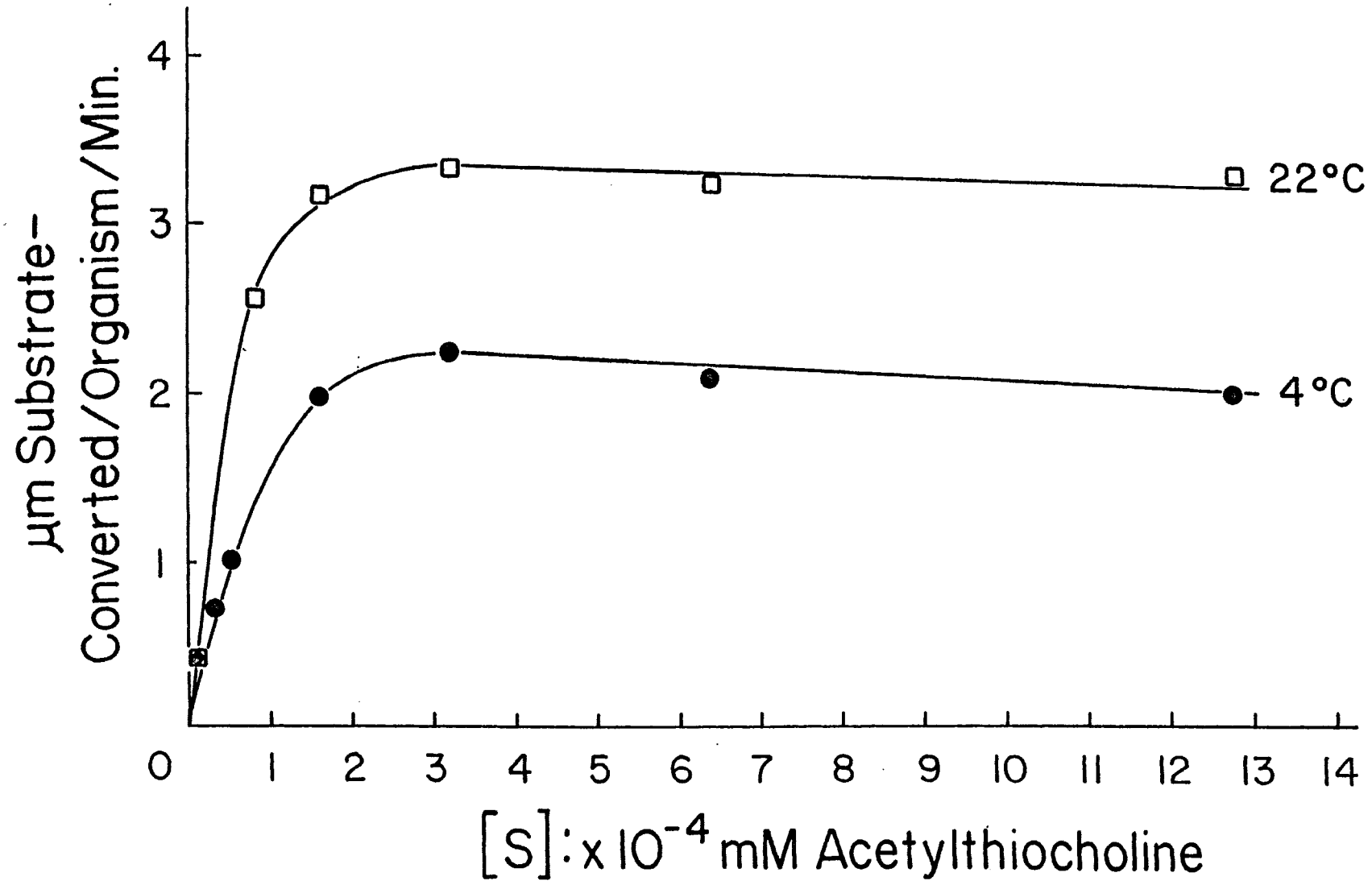
Respiration data (Table 3) did not correlate with toxicity data (Table 4) among the summer forms of these species. While summer respiration rates were very similar, large differences in MFO activity existed among all species. More variation in respiration rates and less variation in MFO activity existed in the winter forms of the species tested. As a result the respiration rates and toxicity data show some correlation in winter forms with higher respiration associated with greater toxicity.

Respiration rate and toxicity did correlate within summer and winter forms of the same species. Although greater toxicity was associated with higher respiration rates, one must consider other physiological changes may have occurred that were not measured and also recognize temperature differences as an important factor.

Acetylcholinesterate Activity

The optimal in vitro substrate concentrations were ascertained for summer and winter forms of each species at 4°C and 22°C using acetylthiocholine. The optimal concentrations were nearly identical among summer and winter forms of each species. Although acetylcholinesterase activity was higher at 22°C than at 4°C (which may be due solely to differences in temperature) however the activity curves for 4°C and 22°C were similar in shape (Fig. 1, using Chaoborus as an example). The optimum substrate concentration was 6.4×10^{-4} mM for all species except Chaoborus sp. which was 3.2×10^{-4} mM. Inhibition of the enzyme due to excess substrate was not observed within the range of concentrations used in this test (Fig. 1).

Fig. 1 Effect of substrate concentration on *in vitro* acetylcholinesterase activity of *Chaoborus sp.* (summer form) at $4^{o}C$ and $22^{o}C$.


µm Substrate-
Converted/Organism/Min.
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
22°C
4°C
[S]: x 10⁻⁴ mM Acetylthiocholine

The activity of acetylcholinesterase at various temperatures was determined for summer and winter forms of all species. The temperature/activity curves were identical for acetylcholinesterase preparations of winter and summer forms of each species. All forms of all species demonstrated fairly high activity over the range of temperatures tested (Fig. 2, using Cheumatopsyche sp. as an example); activity at 4°C was approximately 60% that of 22°C.

The results of the optimal substrate experiments and the experiments on the effect of temperature support the notion that all of these species exhibited immediate rate compensation of their acetylcholinesterases as the seasons changed. This would also indicate that the same cholinesterase isozymes in aquatic insects are present throughout the year.

Inhibition of Acetylcholinesterase

In vitro inhibition experiments were conducted with acetylcholinesterase preparations of summer and winter forms of all species at 4°C and 22°C (Figures 3-13). Paraoxon was used as the primary inhibitor. Experiments conducted with parathion demonstrated that it was a much less effective inhibitor than paraoxon (Figures 3-5, 8, 9, 11-13). This was first noted by Chamberlain and Hoskins (1951). Paraoxon inhibition was greater at 22°C than at 4°C in seasonal forms of all species (Table 5). Inhibition of the cholinesterase, expressed as the I_{50} (molar concentration predicting 50% inhibition) was similar between summer and winter forms of each species at the same temperature. This similarity of inhibition characteristics also corroborates the data presented previously that the same acetylcholinesterase isozymes exist in different seasonal forms of each species. Therefore, immediate rate compensation of acetylcholinesterase is probably the mechanism of adjustment to temperature (seasonal) changes by these species.

There were no correlations of inhibition data with respiration rates or toxicity data among these species except for Allocapnia sp. which had the highest respiration

Fig. 2 Effect of temperature on *in vitro* acetylcholinesterase activity of *Cheumatopsyche sp*.

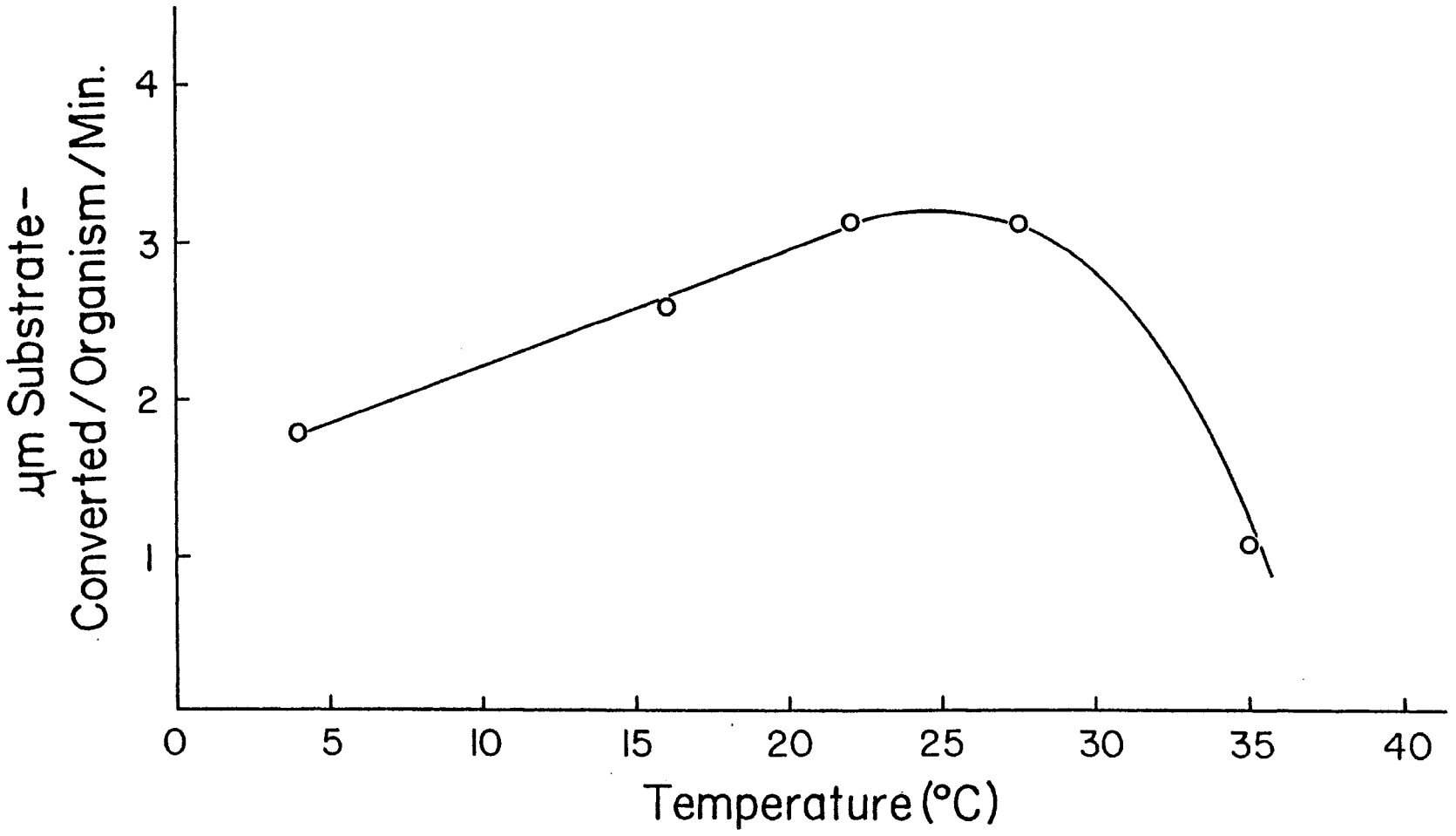

μm Substrate-
Converted/Organism/Min.
1
2
3
4
0
5
10
15
20
25
30
35
40
Temperature (°C)

Fig. 3 Winter form of Allocapnia sp.: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C and by parathion at 22°C.

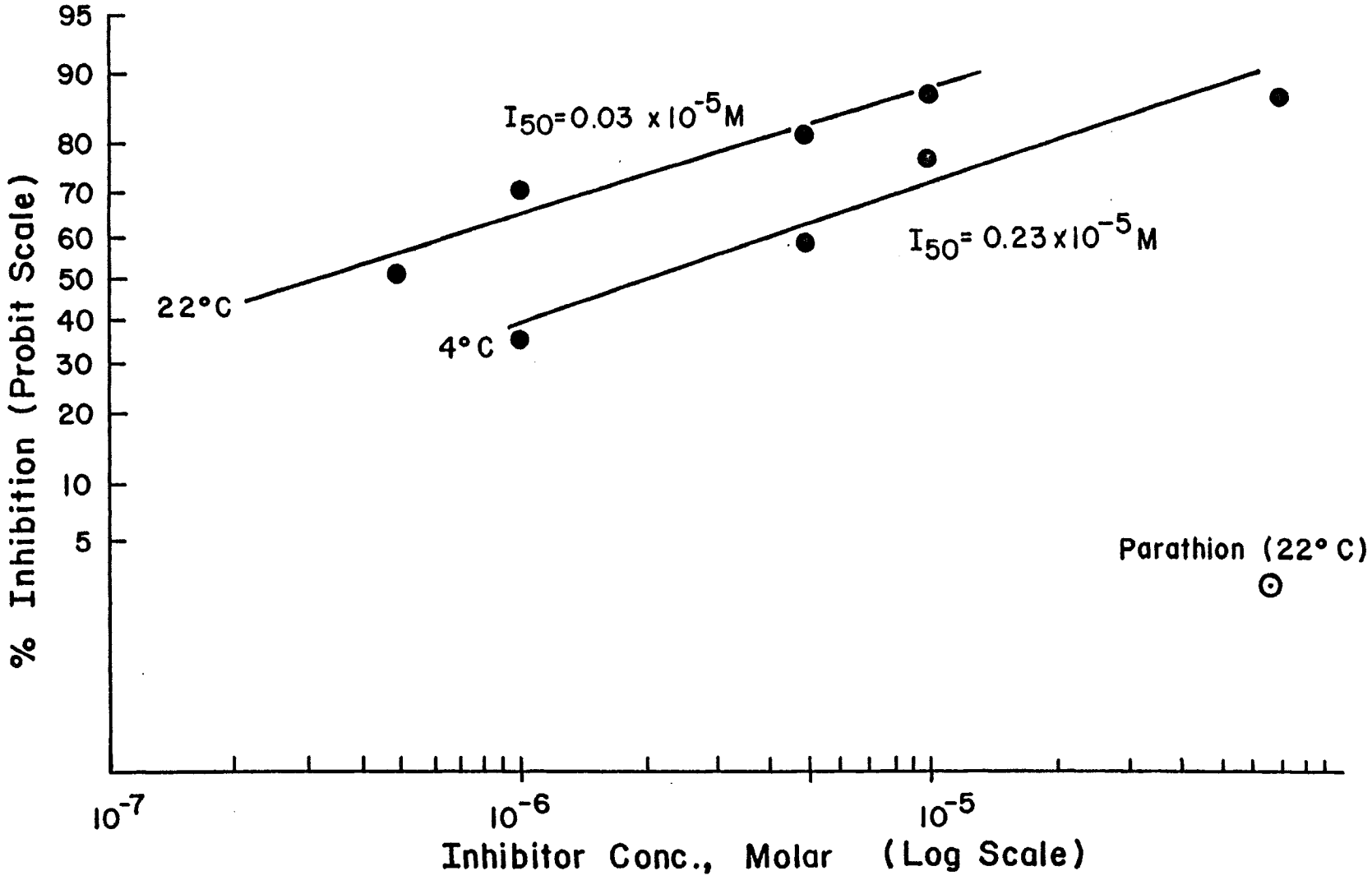

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}=0.03 x 10^{-5}M
I_{50}= 0.23x10^{-5}M
22°C
4°C
Parathion (22° C)
10^{-7}
10^{-6}
10^{-5}
Inhibitor Conc., Molar (Log Scale)

Fig. 4 Summer form of Stenonema femoratum: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C and parathion at 22°C.

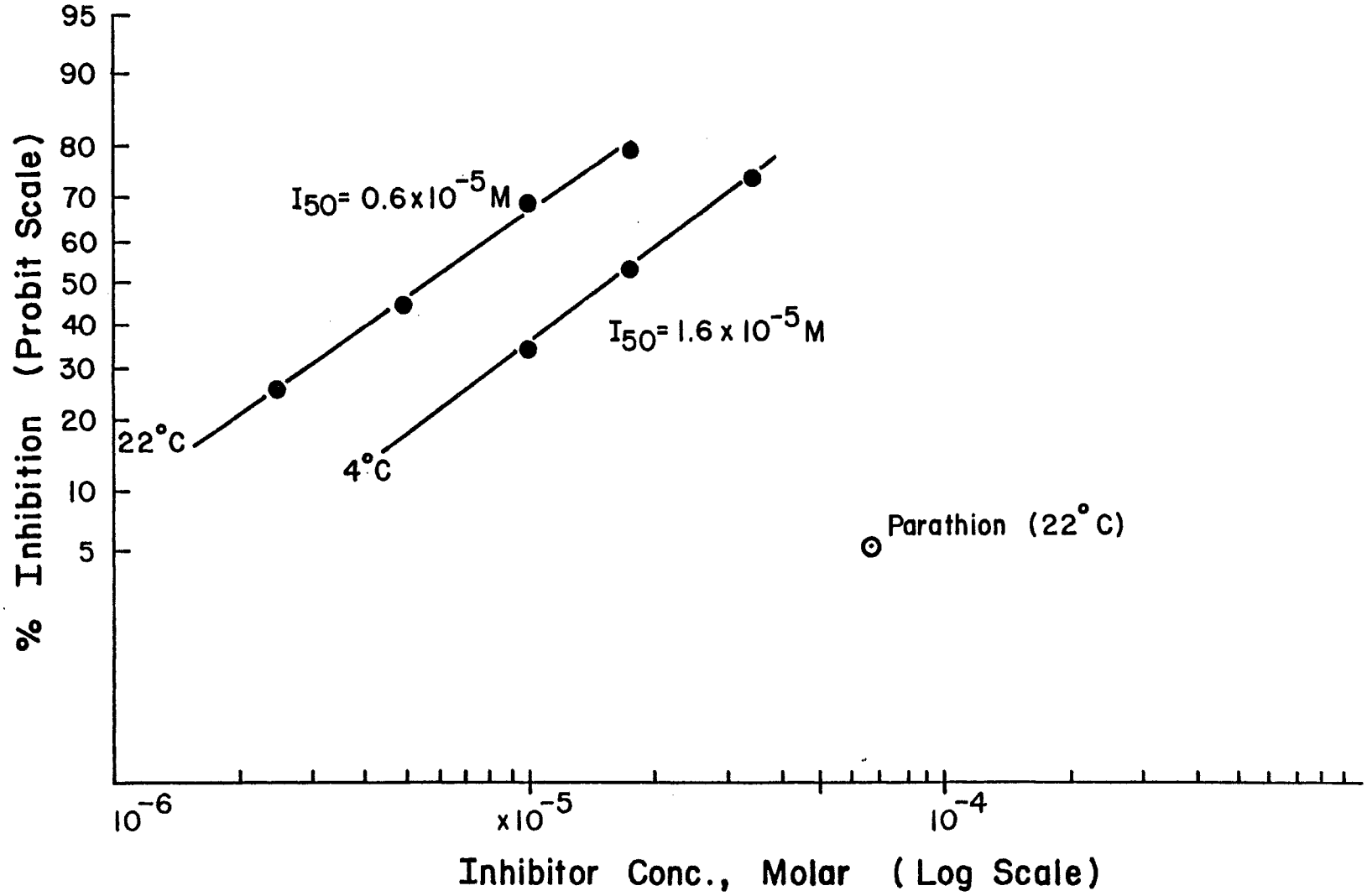

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I50= 0.6x10-5M
I50= 1.6 x 10-5M
22°C
4°C
Parathion (22°C)
10-6
x10-5
10-4
Inhibitor Conc., Molar (Log Scale)

Fig. 5 Winter form of Stenonema femoratum: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C and parathion at 22°C.

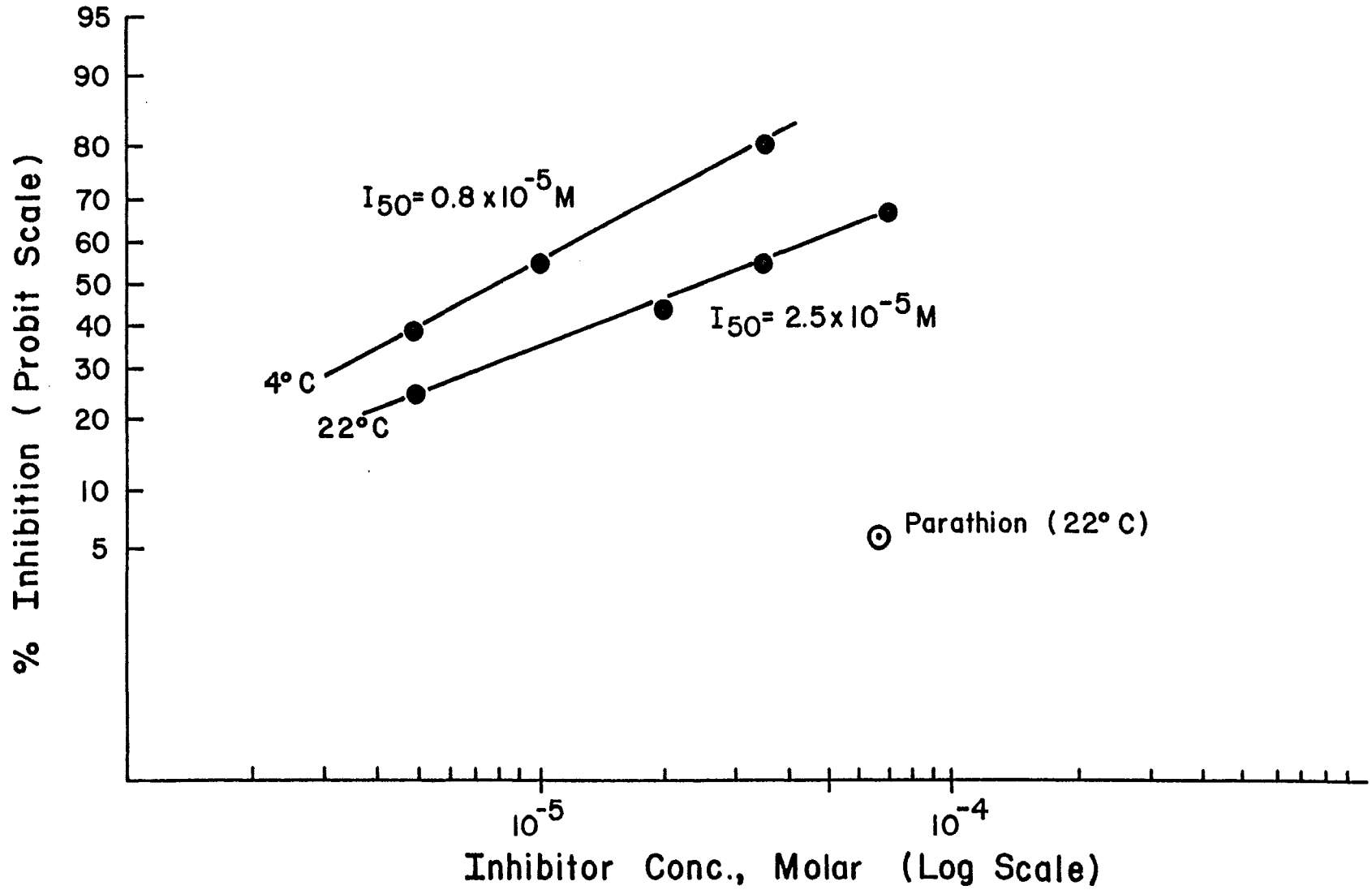

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 0.8 x 10^{-5} M
I_{50}= 2.5x 10^{-5} M
4° C
22°C
Parathion (22° C)
10^{-5}
10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 6 Summer form of Stenonema vicarium: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C.

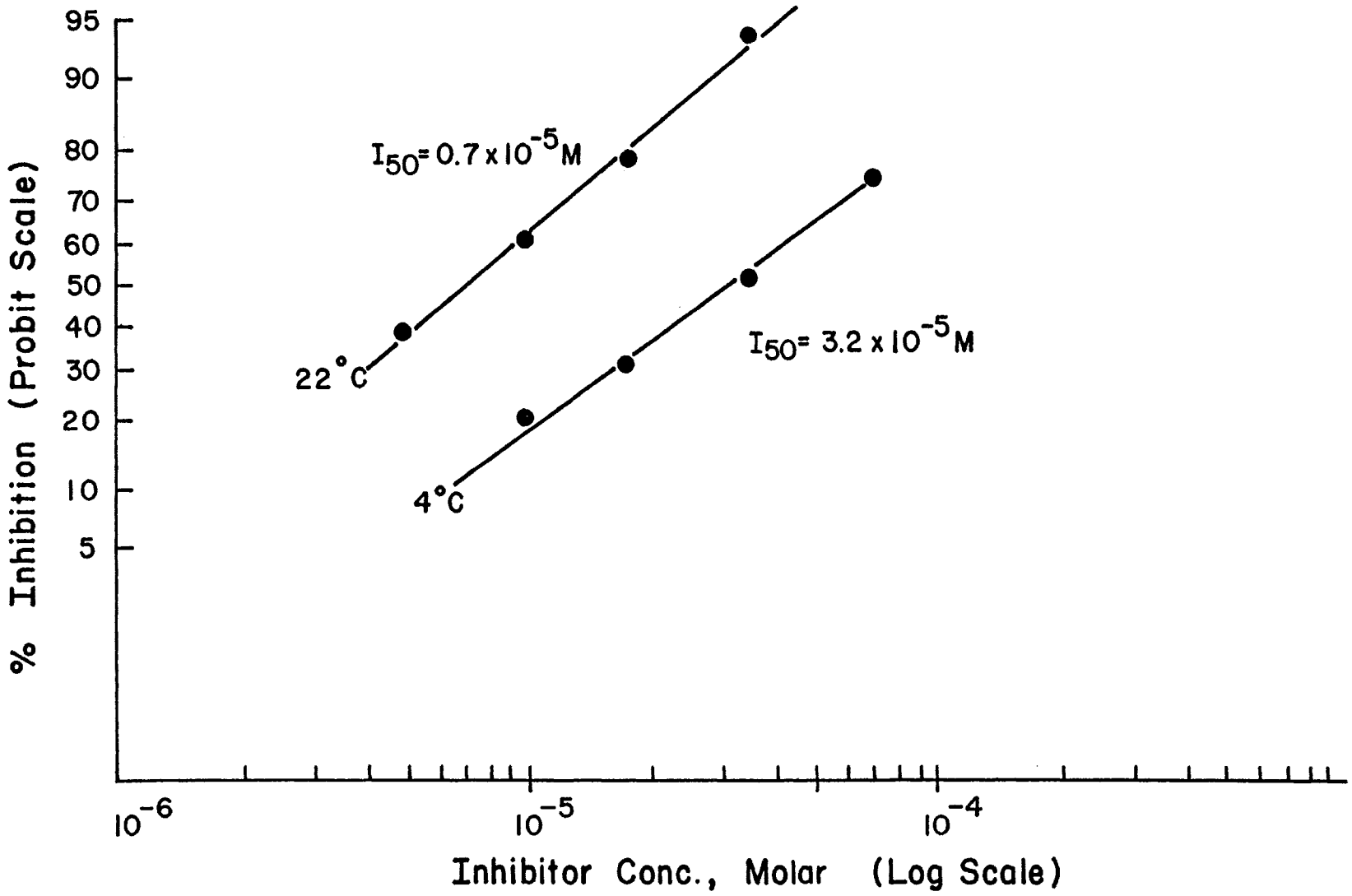

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 0.7 x 10^{-5}M
22°C
I_{50}= 3.2 x 10^{-5}M
4°C
10^{-6}
10^{-5}
10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 7 Winter form of Stenonema vicarium: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C.

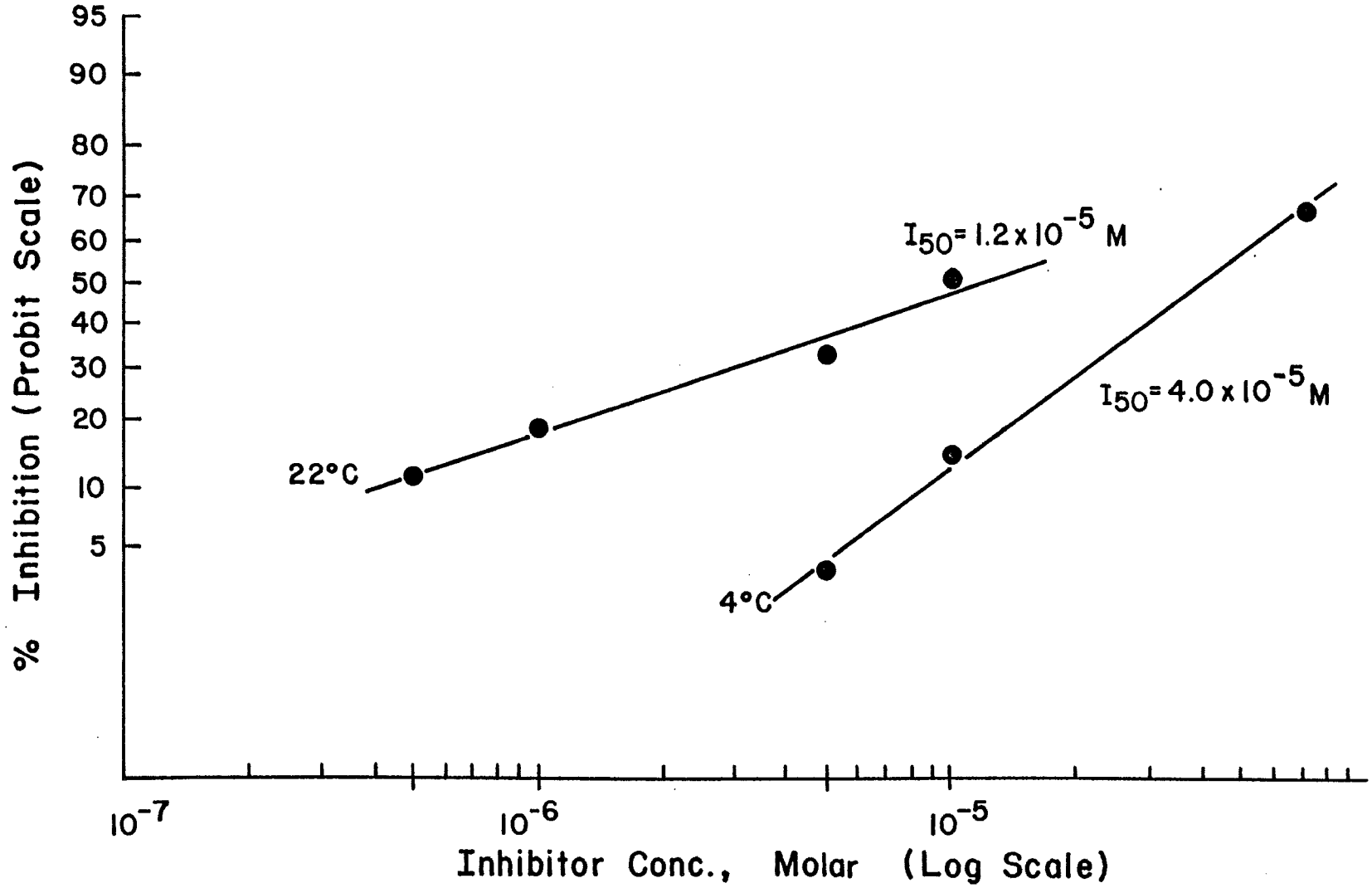

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 1.2 x 10^{-5} M
I_{50}= 4.0 x 10^{-5} M
22°C
4°C
10^{-7}
10^{-6}
10^{-5}
Inhibitor Conc., Molar (Log Scale)

Fig. 8 Summer form of Cheumatopsyche sp.: In vitro inhibition of acetylcholinesterase by paraoxon at $4^{o}C$ and $22^{o}C$ and parathion at $22^{o}C$.

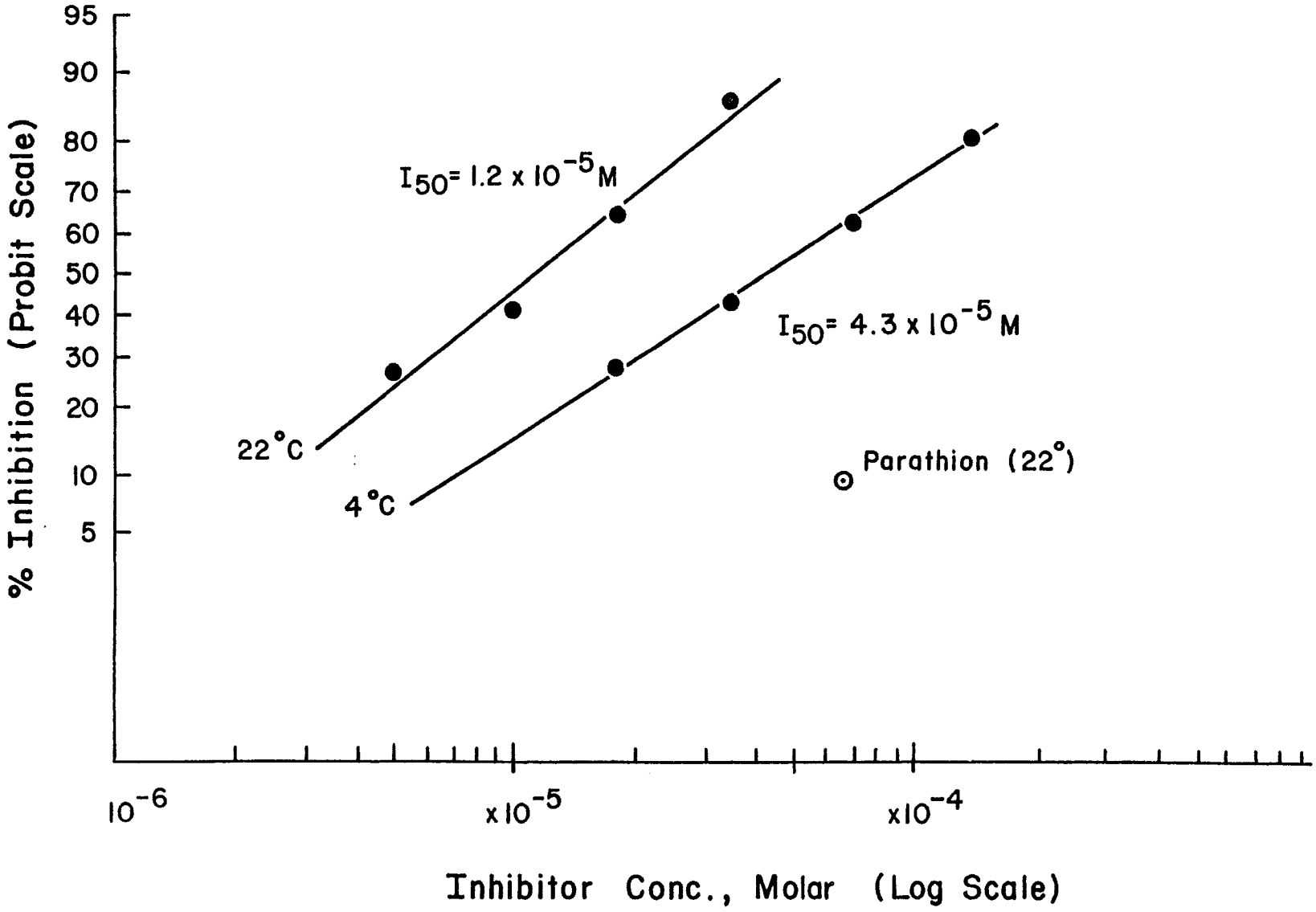

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 1.2 x 10^{-5} M
I_{50}= 4.3 x 10^{-5} M
22°C
4°C
Parathion (22°)
10^{-6}
x 10^{-5}
x 10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 9 Winter form of Cheumatopsyche sp.: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22° and parathion at 22°C.

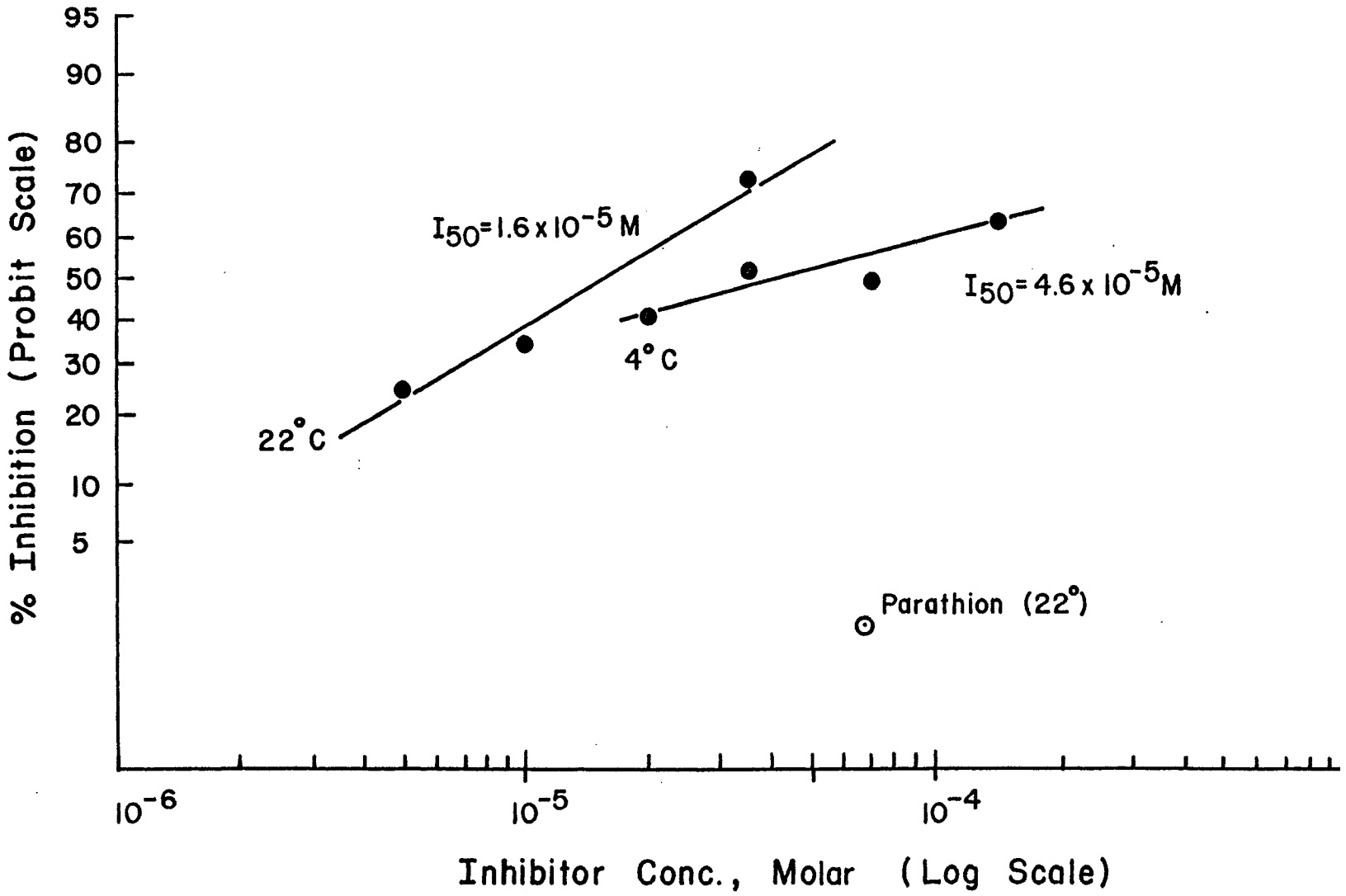

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}=1.6 x 10^{-5} M
I_{50}= 4.6 x 10^{-5}M
22°C
4°C
Parathion (22°)
10^{-6}
10^{-5}
10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 10 Summer form of Hydropsyche sp.: In vitro inhibition of acetylcholinesterase by paraoxon at $4^{o}C$ and $22^{o}C$.

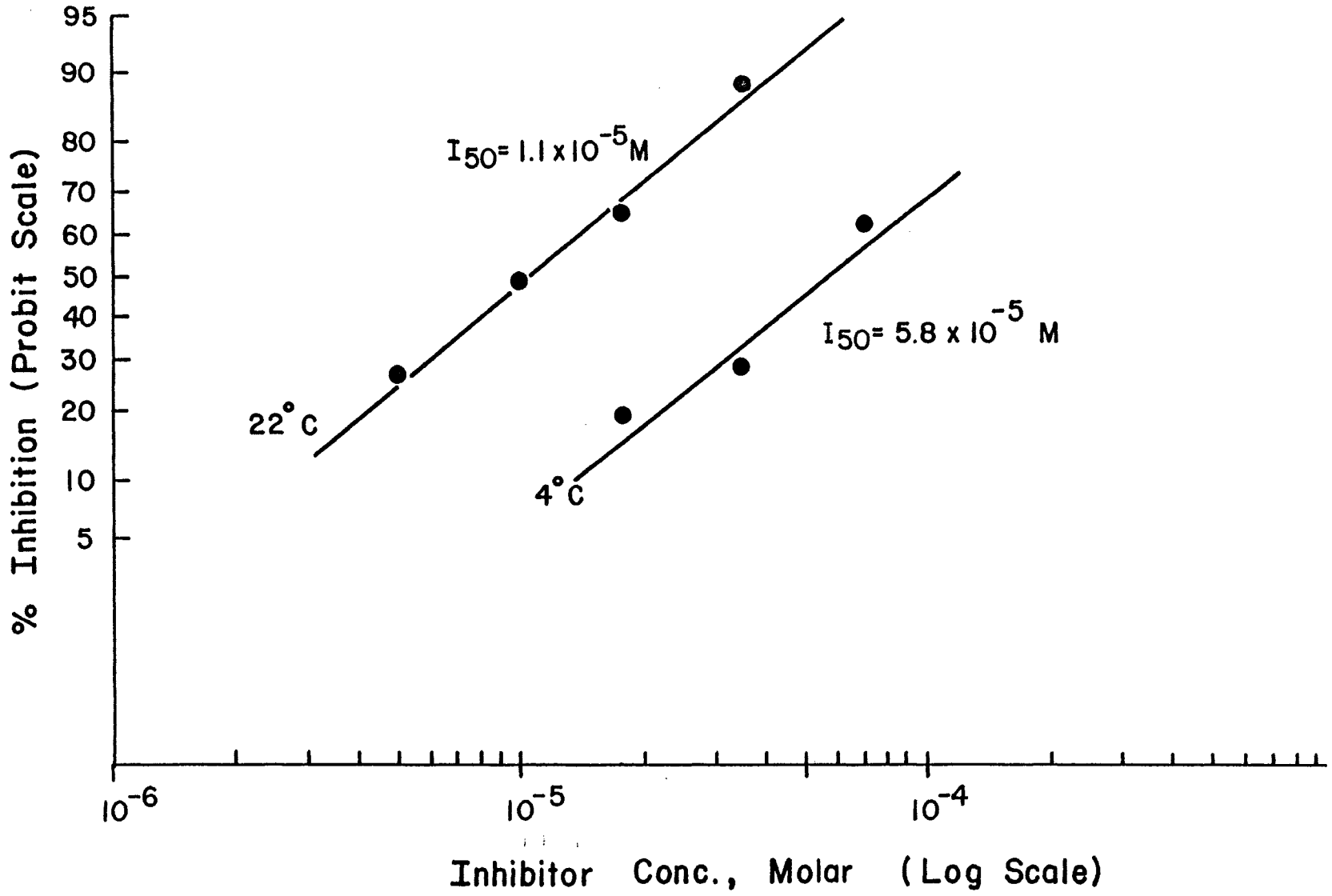

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 1.1 x 10^{-5}M
22° C
I_{50}= 5.8 x 10^{-5} M
4° C
10^{-6}
10^{-5}
10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 11 Winter form of Hydropsyche sp.: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C and by parathion at 22°C.

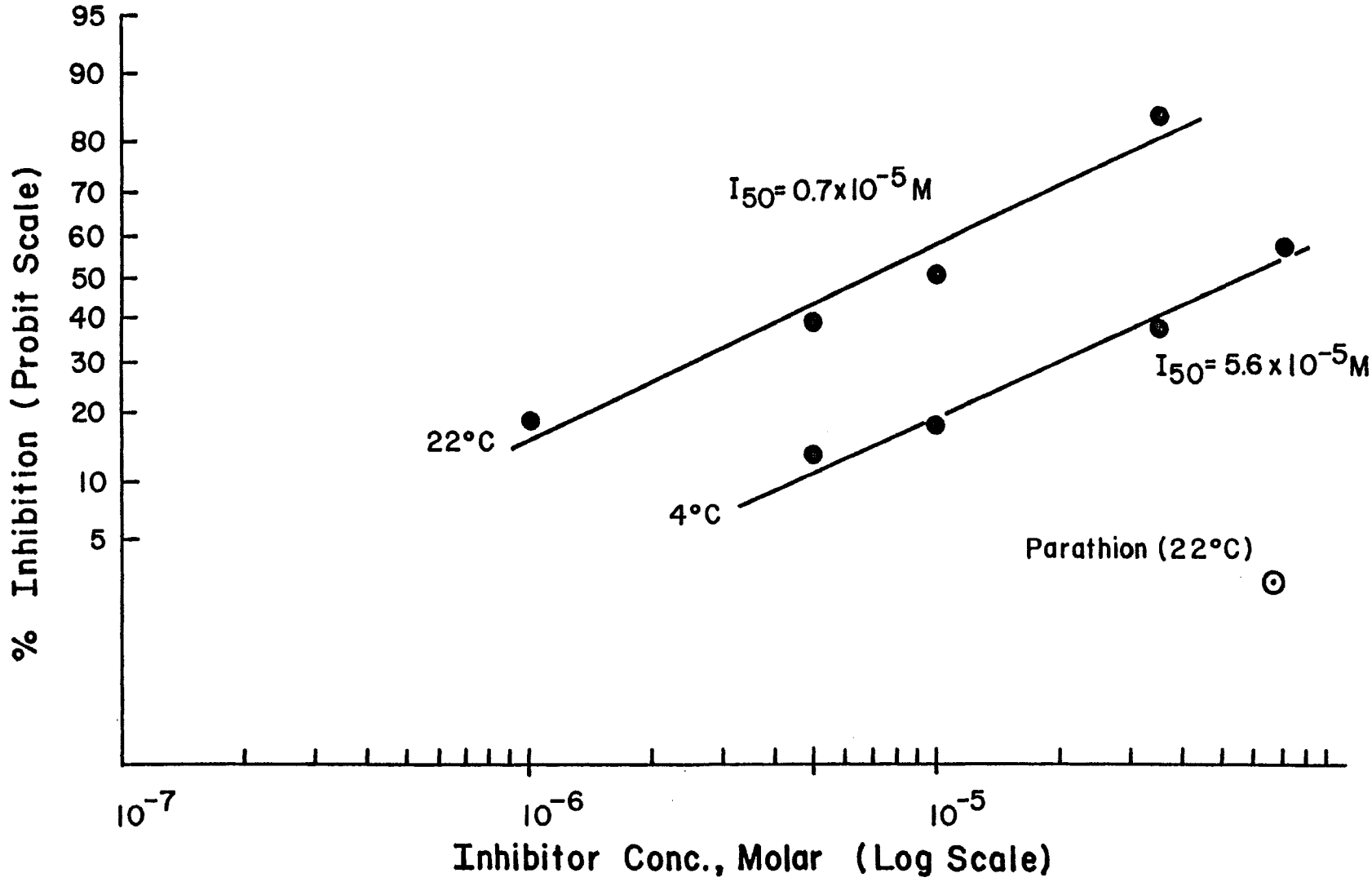

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 0.7x10^{-5}M
I_{50}= 5.6x10^{-5}M
22°C
4°C
Parathion (22°C)
10^{-7}
10^{-6}
10^{-5}
Inhibitor Conc., Molar (Log Scale)

Fig. 12 Summer form of Chaoborus sp.: In vitro inhibition of acetylcholinesterase by paraoxon of 4°C and 22°C and parathion at 22°C.

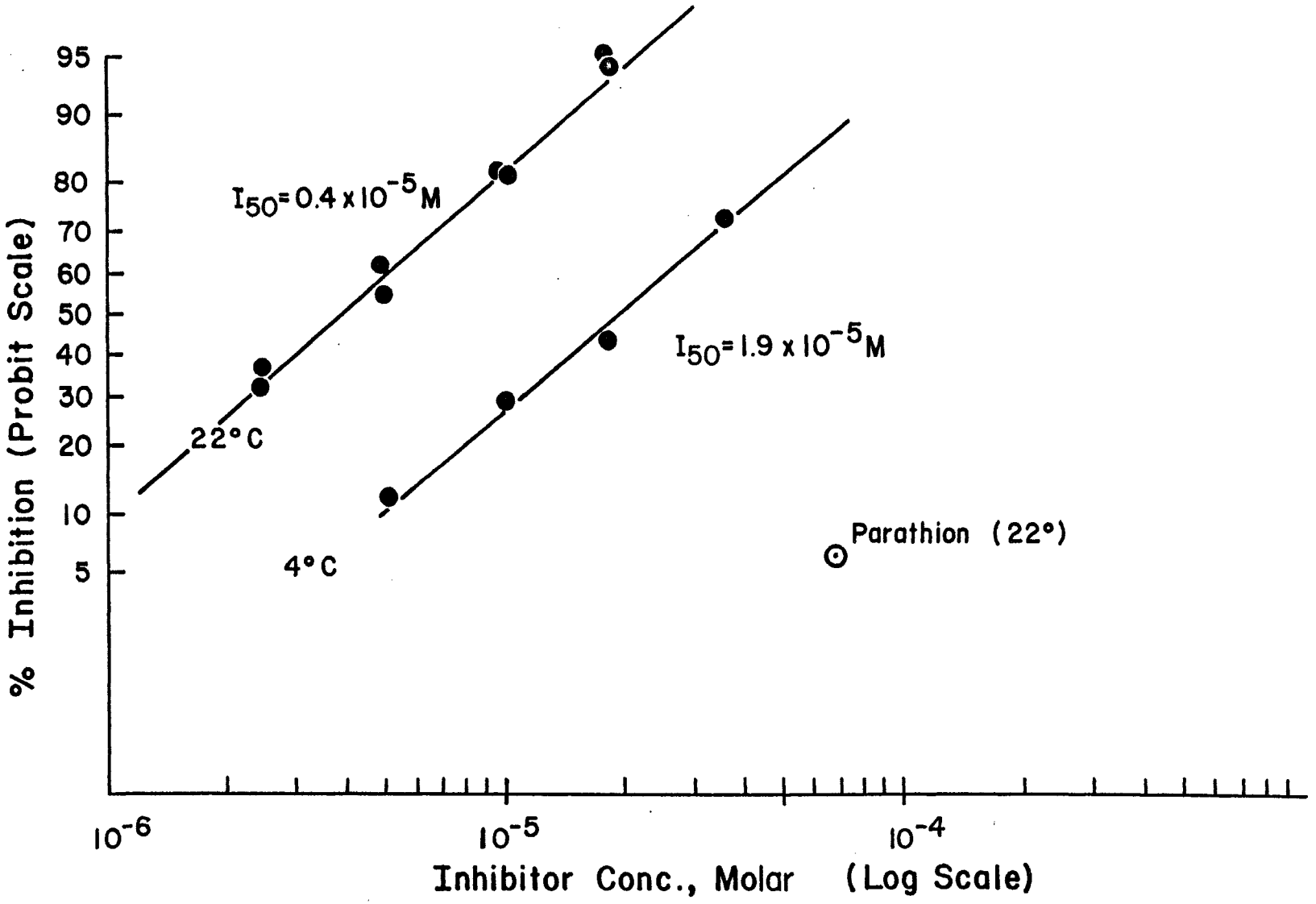
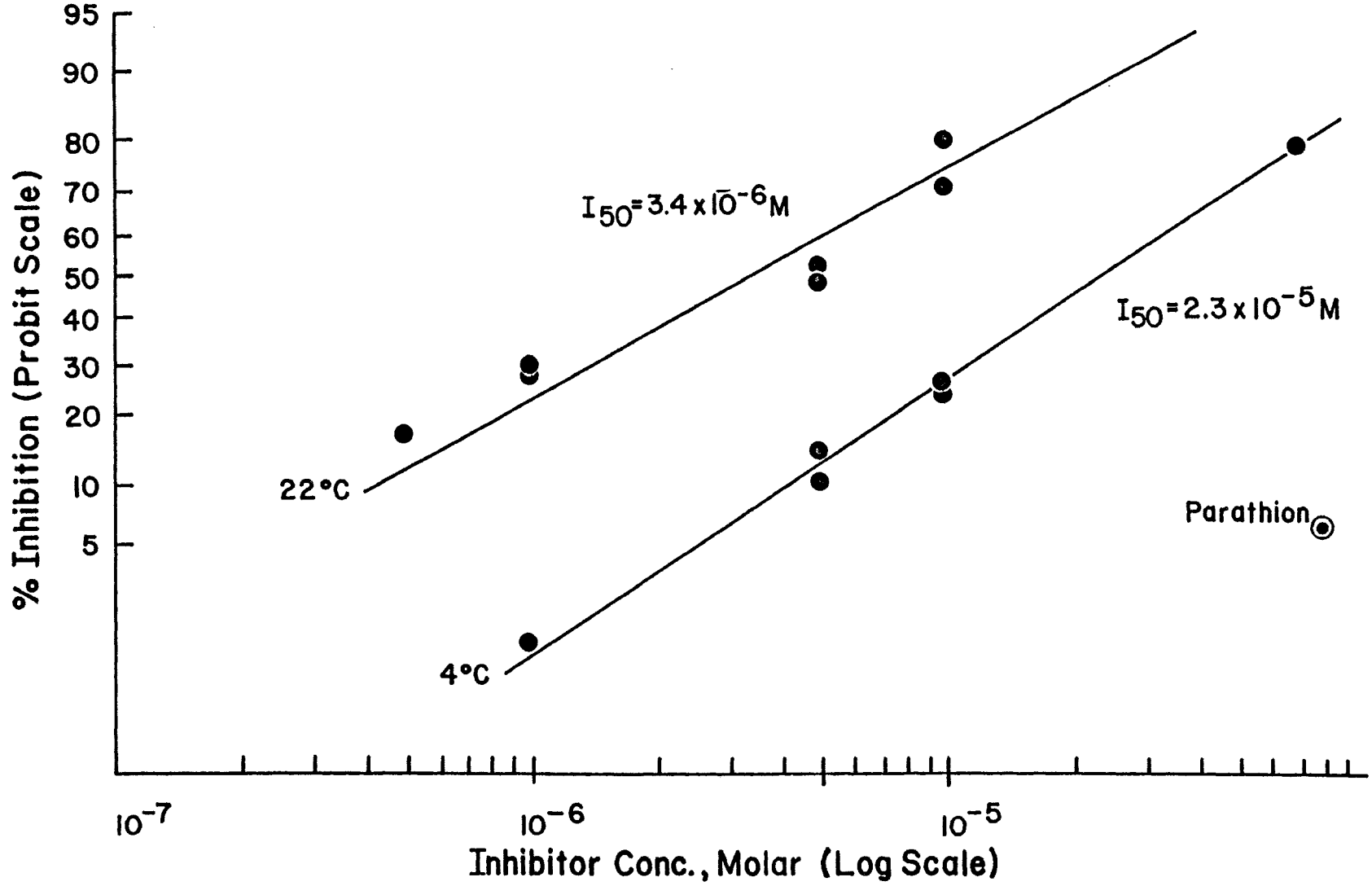

95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
I_{50}= 0.4 x 10^{-5} M
22° C
I_{50}= 1.9 x 10^{-5} M
4° C
Parathion (22°)
10^{-6}
10^{-5}
10^{-4}
Inhibitor Conc., Molar (Log Scale)

Fig. 13 Winter form of Chaoborus sp.: In vitro inhibition of acetylcholinesterase by paraoxon at 4°C and 22°C and parathion at 22°C.


95
90
80
70
60
50
40
30
20
10
5
% Inhibition (Probit Scale)
$I_{50}=3.4\times10^{-6}M$
$I_{50}=2.3\times10^{-5}M$
22°C
4°C
Parathion
10^{-7}
10^{-6}
10^{-5}
Inhibitor Conc., Molar (Log Scale)

rate (Table 3), was the most sensitive organism to the toxicants (Table 4), had the highest MFO activity (interactive toxicity ratio) (Table 4) and the most sensitive acetylcholinesterase (Table 5). This is similar to what Lovell found in mammals (1963) and Morrallo and Sherman with flies (1967). Plecopteran species are well known to be extremely sensitive to pollution (Hart and Fuller, 1974). These findings concur with that general observation.

In vivo inhibition experiments were also conducted with the summer form of Chaoborus sp. using parathion. A linear progression of increasing acetylcholinesterase inhibition occurred as time of exposure to parathion was increased. This was first documented by Metcalf and March (1949). The graph (Figure 14) exhibits the results of two duplicate experiments (100 ug/L parathion) and the tabular results are found in Table 6. The organisms exhibited 6-19% inhibition of their cholinesterase after one-half hour of exposure and 20-40% inhibition after one hour. However, they demonstrated no visible effects at these times despite relatively high inhibition of the enzyme. The first visible signs of toxic effects appeared at two hours and was associated with 41% to 64% inhibition of acetylcholinesterase. Organisms with visible impairment of their ability to move always exhibited 90% or greater inhibition and therefore the percent inhibition in the middle column of Table 6 is always lower than in the last columns where dead or affected animals are present. At 5-1/4 hours, 91% - 95% of the organisms were affected or dead while the few insects that appeared normal exhibited 59% - 83% inhibition of their acetylcholinesterase.

In a companion experiment, organisms were exposed to a high concentration (100 ug/l) of parathion for 1 hour and then transferred to clean water. Although they were showing no effect at this time, 85% were dead within 24 hours. These experiments reaffirm the requirement for a high level of cholinesterase inhibition (80%) to visibly impair the movements of these organisms and suggests that, at high concentrations of parathion (100 ug/l), sufficient parathion accumulated internally

Fig. 14 *In vivo* inhibition of acetylcholinesterase in *Chaoborus sp.* (summer form) exposed to 100 ug/L parathion at 22°C. A and B are duplicate experiments conducted at different times.

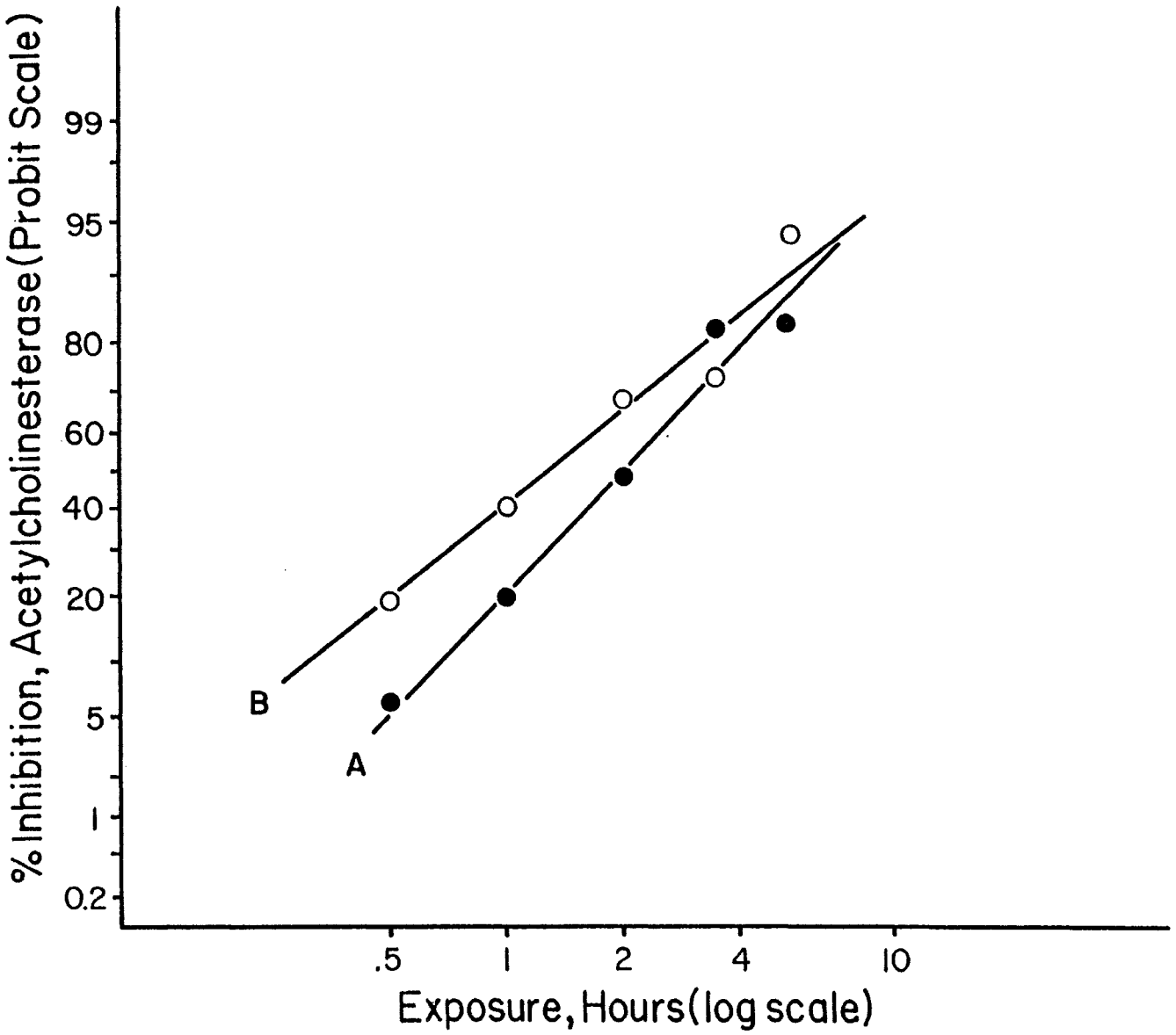

% Inhibition, Acetylcholinesterase (Probit Scale)
99
95
80
60
40
20
5
1
0.2
B
A
.5
1
2
4
10
Exposure, Hours (log scale)

Table 5

In Vitro Inhibition of Acetylcholinesterase by Paraoxon In Experimental Insects: I_{50}, Molar ($X\ 10^{-5}$)

	Summer Form		Winter Form	
	4°C	22°C	4°C	22°C
Allocapnia sp.	-	-	0.23	0.03
S. femoratum	1.6	0.6	2.5	0.8
S. vicarium	3.2	0.7	4.0	1.2
Cheumatopsyche sp.	4.3	1.2	4.6	1.6
Hydropsyche sp.	5.8	1.1	5.6	0.7
Chaoborus sp.	1.8	0.4	2.3	0.35

during one hour of exposure to accomplish a lethal affect after transfer to clean water. Other investigators have also noted the need for high levels of inhibition in order to achieve visible results (Metcalf et al 1949, Chamberlain et al 1951 and Bigley 1966).

Additional experiments were conducted with a lower concentration of parathion (1 ug/l) for a longer period of time (48 hours) (Table 6). None of the organisms exhibited any visible effect or demonstrated any measurable cholinesterase inhibition after 1 hour of exposure. Minimal inhibition (15%) was observed at 12 hours with no visible effect. Visibly affected organisms were observed after 24 hours; unaffected organisms at this time exhibited 20% inhibition. At 48 hours 31% - 67% of the insects were dead or affected; the portion of the exposure group that were visibly unaffected still exhibited only 17% - 24% inhibition of their cholinesterase. Thus, there appeared to be two distinct groups after 48 hours of exposure, those that were dead or affected with 90% or greater cholinesterase inhibition and a group that appeared normal with 20% or less cholinesterase inhibition. An accumulative inhibition effect occurred only in a portion of the insects exposed to the lower concentration of parathion; the other portion of organisms demonstrating no increase in cholinesterase inhibition from 24 to 48 hours evidently had physiological mechanisms that maintained low inhibition at that concentration of parathion.

Experiments were conducted by exposing the summer form of Chaoborus sp. to 1 ug/l of parathion for 24 hours and then transferring the normally appearing organisms to clean water. Dead and affected organisms were counted every 24 hours thereafter and cholinesterase inhibition was periodically determined for representative samples. Parathion is an irreversible inhibitor. The enzyme must be regenerated in order to achieve normal activity (Brady et al 1966). These experiments were intended to determine the length of time for enzyme regeneration to occur.

Table 6

Inhibition of Acetylcholinesterase in Chaoborus sp. (Summer Form) Exposed to Parathion at 22°C

Exposure Time (Hrs)	% Dead or affected		% Inhibition, Normal Insects[a]		% Inhibition, All Insects[b]	
conc. = 100 ug/L	A[c]	B[c]	A	B	A	B
0.5	0	0	6	19	6	19
1.0	0	0	20	40	20	40
2.0	2	11	41	64	48	68
3.5	39	54	76	53	82	73
5.25	91	95	59	83	83	94
conc. = 1 ug/L						
1	0	0	0	0	0	0
12	0	-	15	0	15	0
24	4	37	21	-	24	34
36	12	-	3	-	13	-
48	31	67	24	17	73	69

a. Not visably affected

b. Normal and affected

c. A and B are duplicate experiments

After 24 hours exposure to the parathion, 17% and 23% of the organisms in two separate experiments were either dead or visibly affected (Table 7). At this time, the normally appearing organisms had 8% and 22% inhibition of their cholinesterase. These apparent normal organisms were transferred to clean water and monitored during the next 6 days. There was an additional 12 and 19% dead or affected, respectively, within the next 48 hours but affects did not increase beyond this time. In experiment A, where only 8% inhibition of cholinesterase was observed after the initial exposure, the organisms exhibited no inhibition after 24 hours in clean water and remained unchanged until 120 hours had elapsed, at which time the normally appearing organisms had 22% inhibition.

In experiment B, there was 22% inhibition at the start and it decreased to 0% inhibition within 120 hours. The latter result was to be expected but no explanation for the sudden appearance of inhibition in experiment A after 120 hours can be given. The enzyme apparently regenerates within 5 days at initial inhibition levels of approximately 20%. These experiments were conducted without feeding the organisms. The addition of food may expedite the regenerative process.

Table 7

Acetylcholinesterase Inhibition in Chaoborus sp. (Summer Form) Held in Clean Water at 22°C After Exposure to Parathion

Holding Time (Hrs)[a]	% Dead or affected		% Inhibition Normal Insects[b]		% Inhibition all Insects[c]	
	A	B	A	B	A	B
0	17	23	8	22	23	39
24	11	8	0	<14	7	14
48	19	12	0	11	15	22
72	4	4	0	<11	0	11
96	19	8	0	19	23	27
120	4	8	22	0	25	9
144	8	12	17	0	24	13

a Time held in clean water after 24 hr exposure to Parathion (1 ug/L)

b Not visably affected

c Normal and affected

d A and B duplicate experiments

Conclusions

1. Parathion is significantly less toxic in winter forms of aquatic insects at 4°C than in summer forms at 22°C. This information would support the use of toxicity data acquired at warmer temperatures as the basis for establishing water quality criteria for parathion and other OP insecticides.
2. Paraoxon is less toxic in winter forms of aquatic insects at 4°C than in summer forms at 22°C, but the seasonal toxicity differences are less marked for paraoxon than parathion.
3. Parathion is more toxic than paraoxon in summer forms at 22°C, but in most winter forms, parathion and paraoxon are approximately equitoxic at 4°C. Conclusions nos. 2 and 3 suggest that any environmental transformation of parathion to paraoxon would be beneficial to aquatic insects in warm seasons.
4. Toxicity of parathion varies directly with metabolic rate (oxygen consumption) within a species but toxicity does not correlate with metabolic rate differences between species. Within a species, correlation of metabolic rate are probably due, in most part, to temperature effects on biochemical processes. Besides temperature effects, physiological differences contribute to toxicity differences between species. However, the inhibition characteristics of acetylcholinesterase among five species of insects were not strikingly different.
5. Based on interactive toxicity ratios, oxidative metabolism (MFO) of parathion is significant in some aquatic insects. Inhibiting oxidative metabolism reduced the toxicity of parathion in both summer and winter forms. By the same analysis (interactive toxicity ratios) paraoxon is

not oxidatively metabolized to any significant extent in either seasonal form. In essence, those insects with the highest MFO activity were the most susceptible to parathion. These data suggest that any cocontaminants in natural waters that reduce the oxidative metabolism of parathion would benefit those organisms by reducing the toxicity of parathion.

6. Apparently, the same acetylcholinesterase isoenzyme is functional throughout seasonal changes in the six species. The acetylcholinesterase of winter forms and summer forms is equally susceptible to paraoxon inhibition at the same temperature. In aquatic insects, this enzyme exhibits an immediate rate compensation to temperature changes due to seasonal variation.
7. In Chaoborus, observable toxic symptoms of animals exposed to parathion occur only when high levels of inhibition of acetylcholinesterase occur. With death or observable toxic symptoms as the criteria, some inhibition of acetylcholinesterase may not be harmful to aquatic insects, but these criteria do not include behavioral modification of insects in their natural habitat.
8. Inhibited acetylcholinesterase in exposed Chaoborus is regenerated during 4 to 5 days. Therefore, intermittent insect exposure to parathion in natural waters provides the potential for accumulation of acetylcholinesterase inhibition to harmful levels.

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